Universidade de Aveiro

Departamento de Biologia

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Efeito de nanopartículas de óxido de zinco em *Daphnia magna*

Effect of zinc oxide nanoparticles in *Daphnia magna*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada – Ramo de Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro, Investigadora auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro.

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palavras-chave

Nanotecnologia, nanoparticulas de óxido de zinco, *Daphnia magna*

resumo

O rápido desenvolvimento da nanotecnologia com o consequente aumento na produção de nanopartículas e nanoprodutos oferece muitas oportunidades mas também muitos desafios. A nanotecnologia tem vindo a ser descrita como uma área multidisciplinar que visa desenvolver uma variedade de nanoparticulas para aplicações medicinais e industriais.

As propriedades que trazem às nanoparticulas especial atenção – pequeno tamanho, elevada área de superfície e consequente elevado grau de reatividade – podem também torná-las potencialmente perigosas para a saúde humana e para o ecossistema. A avaliação dos potenciais riscos inerentes à exposição das nanoparticulas torna-se portanto uma investigação de prioridade antes que estas sejam aplicadas em produtos comerciais e libertadas para o ambiente.

Os ambientes aquáticos (de água doce e marinho) são considerados como potenciais destinos das nanoparticulas libertadas para o ambiente através de fontes diretas e/ou indiretas, expondo assim os organismos aquáticos a elevados níveis de contaminação.

As nanoparticulas de óxido de zinco (ZnO-NPs) são uma das nanoparticulas mais utilizadas numa vasta gama de produtos comerciais (ex: protetores solares, cosméticos e tintas) e a sua produção estima-se que irá continuar a aumentar nos próximos anos. Em consequência, o risco de contaminação aquática por parte destas nanoparticulas irá forçosamente aumentar.

Estudos toxicológicos já demonstraram que as ZnO-NPs exercem efeitos tóxicos em vários organismos, como por exemplo, em crustáceos, algas e bactérias. Os efeitos tóxicos das nanoparticulas são complexos e podem estar dependentes de vários fatores, tais como, o organismo-teste, fatores abióticos (pH, salinidade, dureza da água e presença de matéria orgânica), propriedades físico-quimicas das nanoparticulas, processos de adsorção, presença de outros contaminantes, entre outros.

Os objetivos principais deste trabalho consistiram em avaliar a toxicidade das ZnO-NPs com diferentes tamanhos (30 e 80-100 nm) no cladócero *Daphnia magna* e comparar estes efeitos com os homólogos de tamanho micrómetro (ZnO > 200 nm) e a forma iónica ($ZnCl_2$). Os efeitos foram avaliados nos parâmetros de imobilização, inibição alimentar e reprodução.

Os resultados mostraram uma relação dose-resposta entre o decréscimo dos parâmetros avaliados e a concentração das ZnO-NPs, ZnO de tamanho micrómetro e $ZnCl_2$ testadas. De acordo com os resultados obtidos foi possível concluir que o $ZnCl_2$ induziu maior toxicidade aguda para a *D. magna*. Contudo, para a reprodução e inibição alimentar, as nanoparticulas de ZnO mostraram ter um efeito mais tóxico. Foi observado igualmente que o tamanho das nanopartículas não influenciou a toxicidade do ZnO.

Este estudo realça a importância de se estudarem os efeitos de nanoparticulas de diferentes tamanhos uma vez que este é um parâmetro-chave que deve ser considerado quando se pretende estudar a toxicidade de nanoparticulas para o ambiente.

keywords Nanotechnology, zinc oxide nanoparticles, *Daphnia magna*

abstract

The rapid development of nanotechnology with the consequent increase in the production of nanoparticles and nanoproducts presents many opportunities but also many challenges. Nanotechnology has been described as a multidisciplinary field that develops a variety of engineered nanoparticles (ENPs) for medical and industrial applications.

The properties that bring to ENPs special attention for commercial products – small size, large surface area and consequently high degree of reactivity – can also make them potentially harmful for human and ecosystem health. Therefore, assessing the potential risks associated with exposure of ENPs should be considered a major research priority before they are applied in commercial products and released to the environment.

Aquatic (freshwater and marine) environment act as potential destinations for the ENPs released to the environment through direct and/or indirect sources, thus exposing aquatic organisms to high levels of pollutants.

Zinc oxide nanoparticles (ZnO-NPs) are one of the ENPs most applied in a wide range of commercial products (e.g., sunscreens, cosmetics and paints) and its production is estimated to continue to rise in the upcoming years. As a consequence, the risk of aquatic environment contamination by these ENPs will increase.

Toxicological studies have already demonstrated that nanoscale ZnO exert toxic effects in several organisms, such as crustaceans, algae and bacteria. The toxic effects of ZnO-NPs can be complex and may be dependent of several factors such as organism tested, abiotic factors (pH, salinity, water hardness, presence of natural organic matter), physico-chemical properties of NPs, adsorption phenomena, presence of other pollutants in the same environment, among others.

The aims of this work consisted in assessing the toxicity of ZnO-NPs with two different particle sizes (30 and 80-100 nm) in the cladoceran *Daphnia magna*, and compare the results with the respective bulk (ZnO > 200 nm) and ionic ($ZnCl_2$) counterparts.
The effects were assessed based in a set of endpoints: immobilisation, feeding inhibition and reproduction. The results showed a dose-response relationship between all the endpoints assessed and the exposure concentrations of ZnO-NPs, ZnO micro-sized and $ZnCl_2$ tested. According with the results obtained, it was possible to conclude that $ZnCl_2$ induced higher acute toxicity to *D. magna.* However for the feeding inhibition and reproduction endpoints, ZnO nanoparticles showed to exert higher toxicity. In addition it was observed that size did not influence ZnO toxicity. This study highlights the importance to study the effects of nanoparticles with different particle sizes since this is an important parameter to be considered when analysing the toxicity of nanoparticles to the environment.

Index

List of figures and tables

List of figures

List of tables

Chapter 1

General Introduction

1. General Introduction

1.1. Nanotechnology and nanoparticles

Nanoscience is a constant growing research area with a promising future and became a field of scientific interest around the world (Bystrzejewska-Piotrowska et al., 2009; Krysanov et al., 2010).

Nanoparticles (NPs) are known as particles with at least one of their dimensions falling into the nanoscale (1-100nm) (Handy et al., 2008; Klaine et al., 2008; De Berardis et al., 2010; Fabrega et al., (*in press*)), or has a specific surface area by volume greater than 60 m^2/cm^3 according to the European Commission Recommendation of 18 October 2011 on the definition of nanomaterial (http://eurlex.europa.eu/). They can exist in different forms, spherical, tubular, or with irregular shapes (Nowack and Bucheli, 2007).

Nanoscale materials are an intermediate state between bulk and molecular materials (Moore, 2006). The ability to synthesize and manipulate nanoscale materials in this size range has being called of nanotechnology (Nowack and Bucheli, 2007). Nanotechnology presents potential opportunities that enable the synthesis of better materials and products (EPA, 2007).

Since 1990, it has been observed an exponential increase in the development of nanoproducts (products containing nanoparticles) in several industry areas. Some of these applications can bring benefits for human life style because they can be applied for medical purposes of diagnosis, imaging and drug delivery (Nel et al., 2006; Bystrzejewska-Piotrowska et al., 2009).

There are currently many types of nanoproducts applications in the market: electronics, optics, textiles, medical devices, pharmaceutics, telecommunications, cosmetics, food packaging, fuel cells, environmental remediation processes and for catalytic applications (Moore, 2006; Nowack and Bucheli, 2007; Handy et al., 2008).

With the accelerating rate on the production and use of new manufactured nanoparticles (MNPs), the release of NPs into the environment (aquatic, terrestrial and atmospheric) will occur sooner or later (Nowack and Bucheli, 2007). The production estimation of MNPs for 2004 was of 2000 tons and it is expected to increase to 58.000 tons between 2011 and 2020 according to Nowack and Bucheli (2007).

Up to now there is insufficient information about the fate, behaviour and toxicity of manufactured NPs when they reach the environment and if they pose a serious environmental and health threat (Crane et al., 2008; Krysanov et al., 2010) due to contradictory results found in the literature regarding these aspects (Dybowska et al.,

2011). So it becomes necessary, at first, to access the risks of MNPs before they are applied in commercial products to ensure a safe manufacturing and a sustainable nanotechnology industry (Colvin, 2003).

One of the major concerns that has been raised as a consequence of this fast development relates to what is being done to access environmental risks associated with this massive production of NPs for numerous applications and their consequent release of into the environment (Dybowska et al., 2011), as well if this exponential growth of consumer products containing NPs outweighs their many benefits for the society (Colvin, 2003; Barrena et al., 2009).

It has been reported that the development of new technologies implying the use of nanoparticles has been growing much faster than the development of studies that access the implications of these materials to the environment (Krysanov et al., 2010). It should be extremely important to develop at first studies that assess the potential effects of nanoparticles especially to the aquatic environment because natural water bodies are one of the final destinations for nanoparticles through run-off, domestic/industrial wastewaters and also direct release (Baun et al., 2008).

Another concern associated with NPs applications is related to the fact that these days more and more NPs are being used as tools for nanoremediation. Nanoremediation is defined as being the use of inorganic NPs in already polluted environments aiming to reduce and/or eliminate these pollutants (Sanchez et al., 2011). This process involves some advantages (e.g., reduces the costs of clean-up of large scales and eliminates the necessity for other treatments).

However it is also necessary to take into account the costs/benefits of the application of NPs as remediation agents because of the associated risks that are inherent when NPs are applied into the environment (Sanchez et al., 2011). Nanosized materials may not migrate efficiently to be valuable for remediation (Barrena et al., 2009) thus becoming potential health hazards (Johnston et al., 2010). However, many imperative questions remain unanswered because this research area has not yet been completely examined in any great detail.

One main lack of information is coming from the fact that most studies do not present long-term effects associated with the use of NPs for environmentally polluted sites (EPA, 2007; Sanchez et al., 2011).

1.2. Characteristics of nanoparticles

At the nano-scale level, the properties of materials differ substantially from the properties of the respective bulk material of the same composition (Xiong et al., 2011) which from the industrial and medical point of view result in performances with exceptional achievements (Nel et al., 2006). Therefore the use of NPs has obvious advantages (Tomilina et al., 2011).

On the other hand, they can become potentially harmful to the environment and living organisms causing adverse effects if NPs – biologic systems interactions occur (Nel et al., 2006).

According to Heinlaan et al., (2008) the physico-chemical differences between nanoparticles and bulk materials will induce a difference of bioavailability and toxicity of these materials.

The properties that make nano-scale materials an attraction for both industrial and medical applications but also a potential danger to living organisms are:

- Small size and large surface area;

As a consequence of their small size, NPs may become more reactive, due to its large surface area (Elsaesser and Howard, 2011), allowing them to accumulate and/or penetrate in cells and/or organisms more efficiently, possibly causing higher toxicity that would not be possible with the same material in the bulk form (Brayner et al., 2010; Peng et al., 2011). Indeed, NPs of CuO showed to be more toxic than bulk CuO for crustaceans *Daphnia magna* and *Thamnocephalus platyurus* (Heinlaan et al., 2008). However this is not a straight rule because they can form aggregates with sizes comparable with their bulk counterparts which may change their toxic potential (Wong et al., 2010).

- Shape (Bystrzejewska-Piotrowska et al., 2009);

Shape is also a relevant factor for nanoparticle toxicity. For example, carbon nanotubes are known to easily pierce cell membranes thus being able to cause toxicity (Bystrzejewska-Piotrowska et al., 2009). In a case study, Pal et al., (2007) observed that different shaped (truncated triangular nanoparticles, spherical nanoparticles and rod-shaped nanoparticles) silver nanoparticles induced different levels of bacterial growth inhibition to the gram-negative bacterium *Escherichia coli*. Truncated triangular silver nanoparticles were the ones to cause higher biocidal action when compared with the other shaped nanoparticles investigated (Pal et al., 2007).

- Chemical composition (Nel et al., 2006);

Nanoparticle toxicity can be influenced by the chemical toxicity of materials from which they are made, thus making it a call for attention when introducing nanoparticles into the environment (Bystrzejewska-Piotrowska et al., 2009).

- Solubility (Nel et al., 2006);

Solubility is considered a key factor for aquatic toxicity (Kahru and Dubourguier, 2010). For instance, the solubilisation of metal-containing NPs is considered one of the main factors for nanoparticle toxicity (Kahru and Dubourguier, 2010). Indeed, Miao et al.,(2010) described that the toxic effect of ZnO-NPs to the marine diatom *Thalassiosira pseudonana* could be explained by the release of zinc ions to the test medium.

- Aggregation and agglomeration processes (Nel et al., 2006).

Aggregation processes often occur in aquatic environments which may lead to a different behaviour and consequent different impact to the environment (Kahru and Dubourguier, 2010).

In order to measure toxicological endpoints, the properties of nanoparticles need to be fully understood and characterized otherwise the effects of nanoparticles can be wrongly attributed to a certain property of the nanomaterial when the responsible for the effects may come from other pollutant or derived from impurities (Handy et al., 2008; Elsaesser and Howard, 2011).

1.3. Types of nanoparticles in the environment

NPs can be found in the environment resulting from natural and/or anthropogenic sources (Klaine et al., 2008). Non-natural nanoparticles can be produced unintentionally during combustion processes or intentionally produced being designated as engineered NPs (ENPs) (Nowack and Bucheli, 2007).

Natural NPs have always existed among us (Handy et al., 2008) and have been used for millions of years by humankind (Nowack and Bucheli, 2007). Natural NPs have their origin in natural sources such as volcanic ashes, byproducts of combustion fuels (e.g. coal, petroleum and wood burning) erosion processes and others (Handy et al., 2008; Bystrzejewska-Piotrowska et al., 2009). Examples of natural NPs are: organic colloids, such as humic and fulvic acids, and aerosols such as organic acids and sea salts (Nowack and Bucheli, 2007).

Contrary to natural NPs, ENPs show higher levels of complexity with unique physico-chemical features to achieve the idealized properties for the product application.

Examples of ENPs are: fullerenes such as $C_{60,}$ carbon nanotubes (CNTs) and metal oxides, such as TiO_2 and ZnO (Nowack and Bucheli, 2007).

There are different classes of ENPs depending on their chemical properties (Klaine et al., 2008). These classes include: carbon nanoparticles (fullerenes and nanotubes), metal oxides (ZnO, $TiO_{2,}$ Ce_2O_3), zero valent metals (Au, Ag), semiconductors (quantum dots) and nanopolymers (dendrimes) (Klaine et al., 2008). Metal oxide NPs and CNTs are the most produced classes of nanoparticles for commercial and industrial nanoproducts (Klaine et al., 2008; Johnston et al., 2010).

1.4. Nanoparticles-environment interactions

The behaviour of NPs in the environment can be complex and dependent on many processes, like abiotic factors (e.g. pH, salinity, water hardness, presence of natural organic matter), physico-chemical properties of NPs, adsorption phenomena, presence of other pollutants in the same environment, that in return may influence their toxicity (Handy et al., 2008).

Also in the environment, NPs will be in contact with different substances such as small structures (e.g., atoms, single molecules and/or macromolecules), organic natural matter, soil compounds, microbes and others that may enhance the formation of coatings which in turn may modify NPs surfaces and affect their reactivity (Handy et al., 2008).

Moreover, NPs can be released to the environment as free nanoparticles, functionalized nanoparticles, in aggregates or embedded in a matrix (Nowack and Bucheli, 2007; Bystrzejewska-Piotrowska et al., 2009). Once in the environment they can disperse into water, soil or air and act as potential environment hazards by biomagnification in the food chain (Nowack and Bucheli, 2007) affecting many groups of organisms.

Within the cells, due mostly to its small size and consequent large surface area, NPs can display a higher number of reactive oxygen species (ROS) on the surface which is currently the best-developed paradigm of NPs toxicity (Nowack and Bucheli, 2007). This event can cause damages to lipids, carbohydrates, DNA and proteins (Nel et al., 2006; Nowack and Bucheli, 2007; Elsaesser and Howard, 2011). Besides ROS production there are other main causes of nanoparticle toxicity. According to Miller et al., (2010) the dissolution of metal ions from metal oxide nanoparticles can also display an important role

in the toxicity of NPs. ZnO-NPs has already been reported to cause toxicity through these two pathways (Franklin et al., 2007; Xia et al., 2008).

1.5. Behaviour of nanoparticles in the aquatic environment

When it comes to aquatic organisms, there are still some uncertainties about the exposure effects of NPs because it is not yet fully understood the fate and behaviour of NPs in the water column.

For instance, marine environments compared with freshwater environments are more alkaline (higher pH) and present higher ionic strength than freshwater environments (Klaine et al., 2008). As a result, NPs are likely to suffer processes of aggregation (Klaine et al., 2008) being less available to cause toxicity.

Not many studies have addressed the influence of abiotic factors such as pH, ionic strength, NOM and others in the behaviour of NPs in natural environments (Wong et al., 2010), especially in very small nanoparticles (Bian et al., 2011). The results obtained from Bian et al., (2011) showed that different conditions of ionic strength, pH, adsorption of humic acid and particle size affect the aggregation and dissolution of ZnO-NP in aqueous solutions.

Agglomeration and aggregation processes of ENPs can result in deposition of nanoscale particles in sediments (Klaine et al., 2008). However, do to their high reactivity they may not be in this state forever (Lv et al., 2012) and they can be object of geochemical processes that may disperse NPs into the water column (Klaine et al., 2008). Moreover, Yu et al.,(2011) reported that pH values closer to the zero point of charge for ZnO-NPs (9.4 – 9.5) formed aggregates, likely due to reduced repulsive interactions between particles.

Nanoparticles can also suffer action of turbulent waters, a variety of chemicals (detergents, organic matter), be coated by proteins, interact with humic and fulvic acids that may keep them disperse (Klaine et al., 2008).

In a recent study, Lv et al., (2012) reported that elements such as phosphates (major source of contamination in water) play a role in the behaviour of metal oxide NPs in aquatic environments. Due to their high specific surface, hence, high reactivity, metal oxide NPs can interact easily with phosphates influencing their speciation (Lv et al., 2012). The results of their study showed that phosphates were able to reduce the solubility of ZnO-NPs and rapidly caused aggregation (Lv et al., 2012). These findings have significant

environmental implications since toxicity of ZnO-NPs could be greatly reduced in presence of phosphates.

Therefore, taking into account the influence of environmental factors, the behaviour of NPs in different aquatic environments is likely to be different having in return a different impact for aquatic organisms (Klaine et al., 2008).

1.6. Aims and thesis structure

The present work aimed to assess the biological effects of ZnO engineered nanoparticles with different particle sizes to the aquatic invertebrate *Daphnia magna*, since it is reported that the smaller the size, the higher the toxicity of NPs since they are able to interact more easily with organisms. However, as it was reported before, NPs can suffer aggregation processes once they reach the environment. Therefore, to compare the effects of two ZnO-NPs with different particle sizes (30 nm and 80-100 nm) to *D. magna,* we also exposed daphnids to ZnO micro-sized (>200 nm) and $ZnCl_2$. To achieve this purpose, acute and chronic exposures were performed.
In addition, to not exclusively rely to a single aquatic species, it was elaborated a brief overview of the impacts of ZnO-NPs to aquatic organisms.

Therefore the present work was organized as follows:

- A first chapter where concepts related to nanoparticles are presented such as information about their unique features, wide range of applications and behaviour in the environment;
- A second chapter entitled “Effect of zinc oxide nanoparticles in aquatic organisms” where the effects of ZnO-NPs to some aquatic species are reported based in the literature available.
- A third chapter entitled of “Effect of zinc oxide nanoparticles in *Daphnia magna*: size dependent effects and counterparts”. On this chapter the effects of ZnO-NPs were assessed in a broad of several endpoints: immobilisation, feeding inhibition and reproduction.
- The fourth and last chapter provides a general discussion and conclusions of this work.

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Chapter 2

Effect of zinc oxide nanoparticles in aquatic organisms

2. Effect of zinc oxide nanoparticles in aquatic organisms

2.1. Abstract

The increasing use of engineered nanoparticles (ENPs) in industrial and daily life applications will result in the release of these nanoscale materials into environmental compartments.

The aquatic environment is one of the possible and last sinks for any chemical/pollutant that reaches the environment. For this reason, aquatic organisms, especially invertebrates, are widely used in toxicity testing.

It is demonstrated that the behaviour of nanoparticles in the environment is still rather poorly understood, requiring closer attention from regulatory agencies since the release of ENPs into environmental compartments will increase in the very near future.

One of the most produced classes of NPs is the class of metal oxide nanoparticles such as ZnO nanoparticles (ZnO-NPs) due to their application diversity. As a consequence of their wide use they will inevitably reach aquatic ecosystems.

Measurements of environmental concentrations of nanoparticles in the environment are difficult to quantify. However, sophisticated probabilistic methods showed predicted environmental concentrations (PECs) for nano-ZnO in U.S and Europe in the range of $\mu g.L^{-1}$ for waters to $mg.Kg^{-1}$ for soils in different environmental compartments.

Knowing that ZnO-NPs pose a risk for aquatic organisms, the aim of this study was to summarize the present knowledge on the fate, behaviour, uptake routes and biological effects of ZnO-NPs to aquatic organisms.

Lastly, current knowledge gaps are pointed out and brief recommendations for future developments are made.

Keywords: Zinc oxide nanoparticles, aquatic organisms, PECs, toxicity

2.2. Zinc oxide nanoparticles

2.2.1. Applications

ZnO-NPs are considered a versatile and technologically important material (Meulenkamp, 1998) because it can be found in a wide range of applications such as biosensors, electronic materials (Brayner et al., 2010), ceramics, rubber manufacturing, as a fungicide (Naddafi et al., 2011), in wastewater treatments (Wong et al., 2010), coatings, paints (Blinova et al., 2010) and textile industry (Heinlaan et al., 2008).

One of the unique properties of ZnO-NPs relates to its large UV spectrum of attenuation properties (Handy et al., 2008) making them one of the most used nanoparticles in personal care products (e.g., sunscreens, tooth pastes, cosmetics) (Blinova et al., 2010; Xiong et al., 2011). This brings in return great attention because of its high possibility to be released into the environment (Franklin et al., 2007; Wong et al., 2010). Researchers estimated that at least 25% of the total amount of sunscreen applied in the skin is washed away during bathing recreations (Wong et al., 2010).

Since ZnO-NPs are used in a wide range of commercial products, this occurrence leads to an increased interest in the behaviour and possible toxic impacts of such materials in the aquatic environment.

2.2.2. Synthesis of ZnO nanoparticles

There are several physical and chemical methods used for the manufacturing of ZnO-NPs. ZnO-NPs can be synthesized by various approaches including sol-gel processing, homogeneous precipitation, mechanical milling, organometallic synthesis, microwave method, spray pyrolysis, thermal evaporation, mechanochemical synthesis (Hong et al., 2009) and forced hydrolysis in DEG, di(ethylene glycol) medium (Brar et al., 2010).

However it has to be taken into account that NPs tend to aggregate due to its large surface area (as a consequence of their small size) (Hong et al., 2009). So, in order to improve the dispersion of NPs, it is necessary to modify their surfaces (Hong et al., 2009). Examples of dispersants that help to maintain the stability of suspensions of ZnO-NPs are Polyethylene Glycol (PEG), Polyvinylpyrolidone (PVP) (Zhang et al., 2007), tri-*n*-octylphosphine (TOPO), sodium dodecyl sulfate (SDS) and others (Brayner et al., 2006).

2.2.3. Release of ZnO nanoparticles to the environment

The increasing use of nanoparticles will lead to the intentionally/unintentionally introduction of NPs in the environment. NPs can remain in the environment for long periods of time and become potentially toxic to aquatic environments because once they are released they may interact with aquatic surfaces, biological species or experience aggregation and sedimentation processes (Brar et al., 2010).

Due to the diversity of ZnO-NPs and other ENPs applications, they can enter the environment through many pathways by both point and non-point sources (Wiechers and Musee, 2010). The main release sources of ENPs into the environment include accidental spillages from industrial activities (e.g., release of liquid and solid waste streams) and

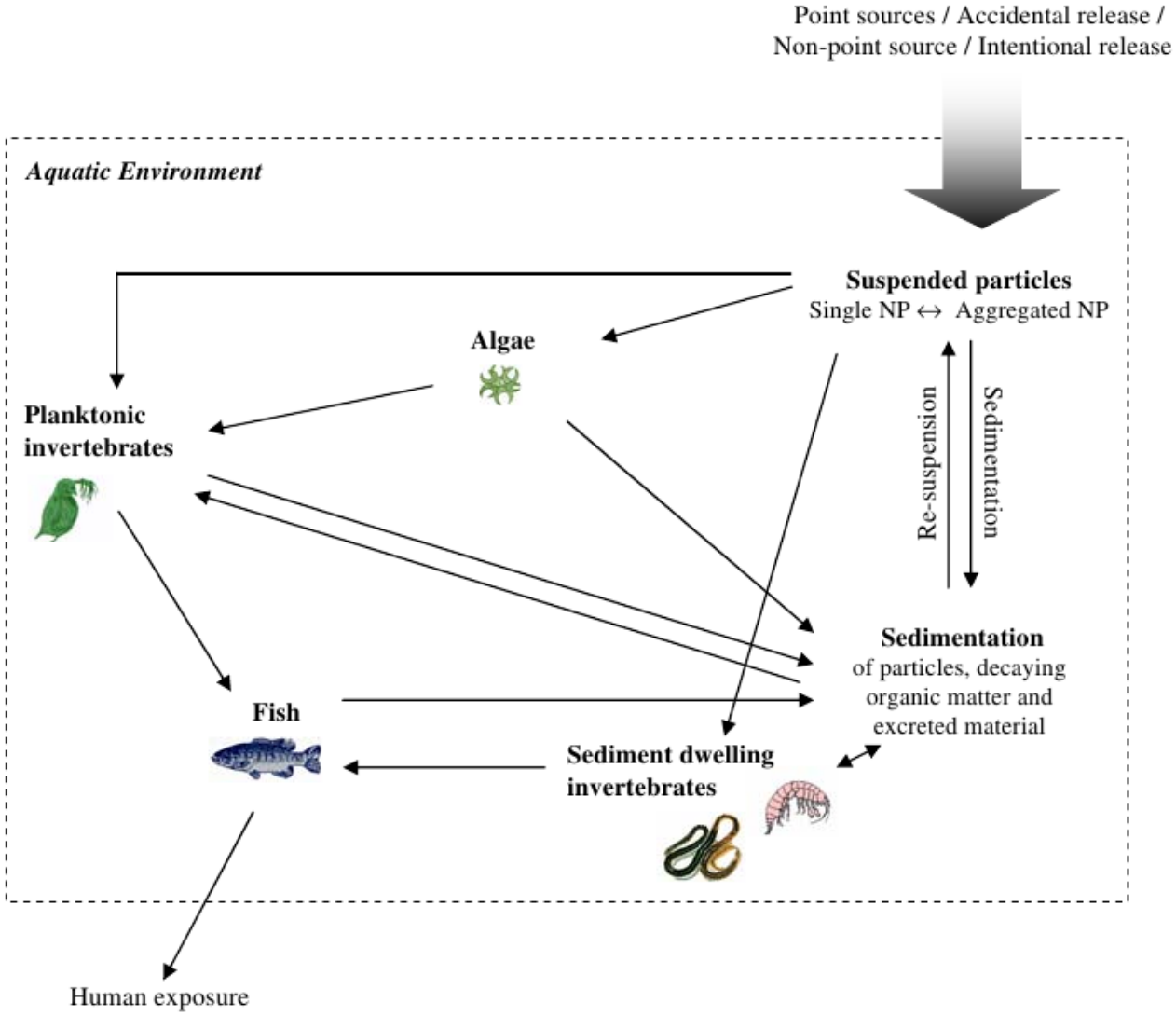

Figure 1.1. Routes of ENPs aquatic environmental exposure and possible interactions with aquatic organisms after their release, *from* Baun et al., (2008a).

during transportation (Wiechers and Musee, 2010). Un-removed NPs from wastewater treatments and agricultural activities (e.g., use of municipal sludge, pesticides) are also sources of release of ENPs into the environment (Wiechers and Musee, 2010).

Figure 1.1 shows the possible routes of ENPs environmental exposure after their release into the aquatic environment. Once they are released ENPs can be suspended in the water column and be taken by planktonic (e.g., daphnids), or by sediment dwelling invertebrates since the sediments are also considered as a potential sink for many contaminants in water ecosystems thus being subject to high levels of contaminants (Baun et al., 2008a).

Given the fact that the release of ENPs from products constitutes a pathway for these nanoparticles to reach the environment, measurements of environmental concentrations of ENPs should be widely examined.

There is few data available reporting the presence of nanoparticles in the environmental compartments, however for ZnO-NPs, Gottshalk et al.,(2009) suggests that several ENPs, including ZnO-NPs may be present in different environmental compartments (Table 1.1).

Table 1.1. Predicted environmental concentrations (PECs) for ZnO-NPs shown as mode (most frequent value) and as a range of the lower and upper quantiles, $Q_{0.15}$ and $Q_{0.85}$, for Europe and U.S. for different environmental compartments (base year, 2008 modified from Gottschalk et al.,(2009). [a] represents the RQ of ZnO-NPs for STP effluents.

	Europe			U.S.			
	Mode	**$Q_{0.15}$**	**$Q_{0.85}$**	**Mode**	**$Q_{0.15}$**	**$Q_{0.85}$**	
Soil	0.093	0.085	0.661	0.050	0.041	0.274	$\Delta\mu g\ kg^{-1}y^{-1}$
Sludge treated soil	3.25	2.98	23.1	1.99	1.62	10.9	$\Delta\mu g\ kg^{-1}y^{-1}$
Surface water	0.010	0.008	0.055	0.001	0.001	0.003	$\mu g\ L^{-1}$
STP effluent	0.432 (10.8)[a]	0.340	1.42	0.3 (7.7)[a]	0.22	0.74	$\mu g\ L^{-1}$
STP sludge	17.1	13.6	57.0	23.2	17.4	57.7	$mg\ kg^{-1}$
Sediment	2.90	2.65	51.7	0.51	0.49	8.36	$\Delta\mu g\ kg^{-1}y^{-1}$
Air		<0.0005			<0.0005		$\mu g\ m^{-3}$

Gottschalk et al., (2009) aimed to calculate predicted environmental concentrations (PECs) of several NPs (TiO_2, ZnO, Ag, CNT and fullerenes) based on a probabilistic material flow analysis from a life-cycle perspective of products containing ENPs. In a second aim, to assess risk quotients (RQs) posed by ENPs, the simulated PECs where compared to the predicted no effect concentration (PNEC) based on ecotoxicological data from the literature for the each environmental compartment.

Table 1.1 shows PECs of ZnO-NPs for air, surface water, sewage treatment plant (STP) effluent and sewage sludge and simulation of the amount of ZnO-NPs deposited in soil, sludge-treated soil and sediment in 2008. For soils and sediments, using estimations of the worldwide market evolution for products containing ZnO-NPs for the period 2001-2012 and assuming zero concentrations in 2000, authors were able to roughly estimate the amount of ZnO-NPs deposited in these compartments for each year of the period considered.

Of all ENPs considered in the study, ZnO-NPs showed to be one of the ENPs with highest concentrations in all compartments with modeled concentrations for natural surface waters of 0.010 $\mu g.L^{-1}$ and 0.432 $\mu g.L^{-1}$ for treated wastewater in Europe. These results reflect that ZnO-NPs may cause significant risks to the aquatic environment.

The study indicated that the risk for aquatic organisms when exposed to ZnO-NPs emanates mostly from sewage treatment effluents due to the fact the risk quotient (RQ) were greater than critical value of one (Table 1.1.) for this compartment but lower to the other compartments (Gottschalk et al., 2009).

Therefore it is suggested that more investigations should be performed in order to evaluate the real risks posed to aquatic organisms by ZnO-NPs.

2.2.4. Routes of uptake and bioaccumulation of ZnO nanoparticles

Uptake of nanoparticles into aquatic biota has been reported to be through direct ingestion and/or entry across epithelial surfaces (e.g., gills, olfactory organs or body wall) (Moore, 2006).

In addition to the uptake, the increase in the concentration of a chemical in a biological organism over time (bioaccumulation) has also to be considered when evaluating potential hazards and risks of NPs since they may become precursors of toxicity (Fabrega et al., (*in press*)). One important question concerning bioaccumulation is whether NPs bioaccumulate after they penetrate into the organism or if they only stay adsorbed to external surfaces causing cell damage.

In a pioneering study with bacteria, Brayner et al., (2006) observed, through TEM images, either accumulation of ZnO nanoparticles in the bacterial membrane of *E.coli* as well as internalization of these nanoparticles as a result of cell wall disorganization. Kumar et al., (2011a) also observed uptake and consequent internalization of ZnO and TiO_2 nanoparticles in *Salmonella typhimurium.*

According to Moore (2006), the uptake of NPs at the cellular level, is thought to be through endocytosis. In endocytosis, molecules or particles between 1 and 100nm are taken up by invagination of the plasma membrane leading to the formation of vesicles that encloses the material and transports it into the cell.

For aquatic invertebrates, Zhu et al., (2009b) studied the toxicity of six NPs (i.e., ZnO, TiO_2, Al_2O_3, C_{60}, SWCNTs and MWCNTs) through an acute bioassay, using immobilisation and mortality as endpoints in *Daphnia magna*.

One of the aims of the study was to create dose-dependency curves for all nanoparticle water suspensions and then to compare with the toxicity of respective bulk counterparts to determine if the size of NPs affected their toxicity to *D. magna*. In addition, the present study also aimed to evaluate the uptake of NPs by *D. magna*.

ZnO-NPs suspension showed to be the most toxic to *D. magna* among all NPs studied with an EC_{50} of 0.622 $mg.L^{-1}$ and an LC_{50} of 1.511 $mg.L^{-1}$.

The uptake of nanoparticles by *D. magna* was recorded by using a microscope with a digital camera (2 x 10 resolution). The authors reported that after 48h of exposure ingestion of NPs and consequent accumulation in the gut occur at the highest concentrations.

There is also available data for other ENPs. For instance, Heinlaan et al., (2011), studied the changes in the midgut of *D. magna*, using TEM technique, when exposed to CuO-NPs and CuO bulk in an attempt to evaluate the size-related effects. TEM is considered an essential tool for (nano)ecotoxicological studies because it enables the visualization of ultrastructural changes of cells and tissues when exposed to nanoparticles (Heinlaan et al., 2011).

At the end of 48h of exposure, CuO bulk particles at a nominal concentration of 175 $mg.CuO.L^{-1}$ (48h-EC_{50}) were found wrapped in aggregates by the peritrophic membrane (TPM). This membrane is designed to protect the gut epithelium and also regulate nutrient and enzyme exchanges (Heinlaan et al., 2011).

Contrary to CuO bulk, CuO-NPs at a nominal concentration of 4 mg.CuO.L^{-1}, corresponding to the 48h-EC_{50} value for CuO-NPs, nanoparticles were taken up by *D. magna* within 10 min of exposure and were found dispersed in the gut of *D. magna* along with bacterial colonization.

Authors suggested that the appearance of bacteria could be caused by suppression of the immune response of *D. magna*. Since no bacterial colonization took place during exposure of *D. magna* to CuO bulk it is suggested that the presence of bacteria observed during exposure of CuO-NPs can be a specific mode of toxicity induced by these nanoparticles.

Taking into account the studies mentioned above, their observations come to reinforce the fact that the potential impacts of ENPs for aquatic organisms should not be neglected since exposure of these nanoparticles may pose a risk of bioaccumulation which may lead to dead of organisms such as filter-feeding organisms (e.g., *Daphnia magna*) affecting like this the balance of the aquatic environment.

Another exposure pathway that may lead to the uptake of ENPs by aquatic organisms can be the through the sorption of NPs to algae. For instance, Croteau et al., (2011) showed the behaviour of the freshwater snail, *Lymnaea stagnalis*, when exposed to well-characterized isotopically modified ZnO-NPs (^{67}ZnO nanoparticles).

The aims of the study were to address the uptake rates of ^{67}Zn in the form of ZnO-NP when ingested with food and the uptake rates of ^{67}Zn also when ingested with food. Benthic diatom *Nitzschia palea* was used as food source.

Whether the form of ^{67}Zn, the results showed that both forms were efficiently assimilated by *L. stagnalis* showing that ^{67}Zn from ZnO-NPs appear equally bioavailable as the ionic ^{67}Zn.

In another phase of the study, authors observed that high concentrations of ZnO-NPs caused interference in the feeding rates of L. *stagnalis* and also affected their defecation rates at high concentrations (10^6 nmol g^{-1}) of ZnO-NPs, thus showing evidences of dietary metal stress. For the authors the reduced feeding activity observed by *L. stagnalis* remained unclear if it was due to high concentrations of Zn (ionic) derived from ZnO-NPs or due to the ZnO-NPs themselves.

To date, many studies have focused especially on the direct effect of NPs. However, NPs can also act as contaminant carriers for other bioavailable toxicants (e,g., metal-containing NPs, C_{60}) (Baun et al., 2008b; Croteau et al., 2011).

It is known that metal-containing NPs are already widely used in consumer products. Therefore, after their discharge to the environment, these NPs may interact with other pollutants (Baun et al., 2008b). This interaction may lead to a change on the bioavailability of these pollutants to aquatic organisms (Baun et al., 2008b) making it a point of concern in the future.

For instance, in a recent study, Naddafi et al., (2011) studied the bioavailability of phenanthrene adsorbed to ZnO-NPs using *Daphnia magna* as the organism test.

The results showed that the bioaccumulation of phenanthrene in *D. magna* was enhanced by the presence of ZnO-NPs when compared with phenanthrene free of ZnO-NPs since its toxicity for 24h and 48h was 1.7 and 2.1 times higher, respectively (Naddafi et al., 2011).

Therefore, and based on the previous study, when it comes to risk assessments of nanoparticles, it is then important to consider not only the inherent toxicity of NPs but also the possible interactions between NPs with already existing environmental contaminants.

Also, the increasing release of ENPs into the environment, their low biodegradability and possible association with the feeding habits of aquatic invertebrates, calls the urge for faster development studies to better understand the behaviour and mechanisms of uptake/bioaccumulation of ENPs in aquatic ecosystems because as described in this section ENPs are easily taken up by these organisms which in the worst cases may lead to death.

2.2.5. Effects of ZnO nanoparticles to aquatic organisms

The impacts of common metals for the health and the environment are well known (Brar et al., 2010). However, when metals take the form of nanoparticles, the potential hazards due to their shape and size are yet to be explored (Brar et al., 2010).

Many studies have already investigated the toxicity of ZnO-NPs on several aquatic organisms (Table 1.2), however the toxic mechanisms are still unclear (Bai et al., 2010).

In studies where the toxicity and interactions of ZnO-NPs with aquatic organisms are assessed, they are in most of the cases compared to either larger ZnO-NPs (ZnO micro-sized) or to ionic Zn (Franklin et al., 2007; Heinlaan et al., 2008; Zhu et al., 2008; Aruoja et al., 2009; Wiench et al., 2009; Zhu et al., 2009a; Zhu et al., 2009b; Wong et al., 2010; Fairbairn et al., 2011; Yu et al., 2011). For metal-based nanoparticles it is important to take into consideration the solubility of ions on their toxicity since many studies have

considered Zn ions as one of the main key factors that accounts for the toxicity of ZnO-NPs to aquatic organisms (Franklin et al., 2007; Heinlaan et al., 2008; Aruoja et al., 2009).

ZnO nanoparticles are widely incorporated in commercial merchandise. However, their environmental impact and their mechanisms of toxicity are not yet fully understood. According to the available literature the toxicity of ZnO-NPs to aquatic organisms is in some part attributed to its solubility in water but also with ROS production even if the this last one still remains a open question (Bai et al., 2010; Miller et al., 2010).

In the next section, several studies concerning the effects of ZnO-NPs to aquatic organisms of different trophic levels of the food chain will be presented.

2.2.5.1. Toxicity to algae

For algae species the endpoint most assessed is the growth inhibition endpoint (Table 1.2.).

Franklin et al.,(2007) developed a study where it presented the effects of ZnO-NPs (nominal particle size of 30 nm), ZnO bulk and $ZnCl_2$ in the growth rate of the freshwater algae *Pseudokirchneriella subcapitata.* The results showed that the toxicity of ZnO-NPs did not present statistical differences when compared with ZnO bulk and $ZnCl_2$, showing 72h-IC_{50} values of 68, 63 and 61 µg $Zn^{2+}.L^{-1}$, respectively.

In addition, concentrations of dissolved Zn^{2+} ions from ZnO-NPs were determined by equilibrium dialysis, with a pore size of about 1 nm (permeable to Zn^{2+} ions but not to ZnO particles) and compared with the concentration of dissolved Zn^{2+} ions from bulk ZnO and $ZnCl_2$. The results showed that at pH 7.6 rapid dissolution rate for both ZnO-NPs and ZnO bulk occurred within 6h yielding similar dissolved zinc concentrations. At the end of the 72h of the experiment, 19% of the nominal concentration (100 $mg.L^{-1}$) of ZnO-NPs and ZnO bulk was dissolved. Taking into account the dialyzed zinc concentrations obtained for all zinc compounds during the experiment, the 72h-IC_{50} were calculated. Once again the 72h-IC_{50} values showed similar toxicities.

Therefore authors suggested that toxicity of ZnO particles (ZnO-NPs and bulk ZnO) as well as $ZnCl_2$ to *P. subcapitata* could be essentially due to dissolved zinc.

In a similar study, Arouja et al., (2009) also investigated the toxicity of ZnO-NPs (50-70 nm particles size) to *P. subcapitata* and aimed to clarify if the influence of particle size and Zn^{2+} ions dissolution played an important role for the toxicity by using other zinc compounds (bulk ZnO and $ZnSO_4$).

Both ZnO-NPs and bulk ZnO showed to be toxic to *P. subcapitata* at low concentrations (<0.1 mg $Zn^{2+}.L^{-1}$) and caused 100% of inhibition at concentrations of 0.16 mg $Zn^{2+}.L^{-1}$. As it happened in the previous study, the toxicity of ZnO-NPs and bulk ZnO showed similar toxicity (no statistical differences regarding the particle size). However, bulk ZnO showed to cause a slightly higher inhibition effect to *P. subcapitata* than ZnO-NPs with 72h-IC_{50} values for ZnO-NPs and ZnO bulk of 0.042 and 0.037 mg $Zn^{2+}.L^{-1}$, respectively.
Regarding $ZnSO_4$, the IC_{50} value (calculated on metal basis) for this compound was of 0.042 mg $Zn^{2+}.L^{-1}$. Therefore and taking into account a previous study developed in their laboratory which indicated that at already low concentrations (0.1 mg.L^{-1}) of both nano and bulk, a high fraction (between 69% and 97%) of Zn (ionic) was already bioavailable, they attribute the toxicity of ZnO-NPs to dissolved zinc ions.

Peng et al., (2011) studied the influence of different sized and shaped (sphered and rod-shaped particles) ZnO-NPs on the growth of three marine diatoms, *Thalassiosira pseudonana, Chaetoceros gracilis and Phaeodactylum tricornutum*, as it has been shown that dissolution reflected as toxicity, is influenced by nanoparticles' morphologies. The particles' dimension analysed by TEM showed that nZnO spheres ranged from 6.3 nm to 15.7 nm and nZnO rod-shaped ranged between 242 nm to 862 nm.
The extent of dissolution in seawater, measured by GFAAS (graphite furnace atomic absorption spectrometry), showed that the solubility of Zn in sphered NPs was higher (but not statistically different) when compared with rod-shaped NPs.
These observations are probably due to the differences in NPs morphology because, according to Borm et al., (2006), smaller particles due to their surface curvature and thinner diffusion layers, will reach faster equilibrium dissolution rates.
They also observed that an increase of the concentration of ZnO-NPs did not result necessarily in an increase of the amount of Zn ions in solution at equilibrium, which allowed the authors to conclude that ZnO-NPs particle concentrations did not have much influence in the dissolution behaviour of nanoparticles probably due to the observation of aggregation of NPs during the experiment.
Regarding the toxicity of ZnO-NPs to the algae species, growth of all marine species was affected by all the concentrations and all different morphologies of ZnO-NPs tested, with *P. tricornutum* being the less sensitive exhibiting a slow but continuous growth rate in the presence of ZnO-NPs. The differences in the sensitivity are explained by the fact that *P. tricornutum* has on its morphology frustules containing less silica than those of the other marine algae species tested. Silica is an important component of diatoms cell wall that

influences their growth. However, even if Zn inhibited diatom growth and frustule formation in *P. tricornutum,* this diatom specie needs less quantities of Si to fulfill its requirements for the formation of its frustule when compared with the other diatom species tested. Therefore, *P. tricornutum* was able to exhibit continuous growth even if in a slow rate.

2.2.5.2. Toxicity to aquatic invertebrates

According to legislative organizations (e.g. REACH), the number of vertebrate animals in toxicological tests should be reduced and alternative testing approaches should be used. Therefore, aquatic invertebrates are being the model organisms most used for (nano)ecotoxicological studies since they represent an important level in the food chain of marine and freshwater ecosystems (Baun et al., 2008a). For aquatic invertebrates the immobilization endpoint is the most common endpoint studied.

There has been a wide range of results concerning the toxicity of ZnO-NPs to aquatic invertebrates.

For instance, Heinlann et al., (2008) using a recombinant Zn-sensor bacteria to compare the toxicity of Zn^{2+} (in the form of $ZnSO_4$), nano ZnO and bulk ZnO, showed that the effect of all Zn compounds for crustaceans *Daphnia magna* and *Thamnocephalus platyurus* were similar (Table 1.2), attributing this to the concentration of soluble Zn^{2+}.

Contrary, Zhu et al., (2009b) also studied the effect of ZnO-NPs in *Daphnia magna* (Table 1.2). However, the value of 48h-LC_{50} (1.511 $mg.L^{-1}$) obtained was higher than the 48h-LC_{50} value (3.2 $mg.L^{-1}$) for ZnO-NPs reported by Heinlaan et al.,(2008).

The differences of the results from both studies could be explained by different approaches adopted in the ecotoxicological protocols since the experimental setup of the first study was performed in the dark which could have influenced the toxicity of ZnO-NPs to the organisms.

In a more recent study, Zhao Hai-zhou., (2012) assessed the effect of ZnO-NPs on the survival, reproduction and feeding behaviour of *D. magna*. The results obtained were in accordance with the study reported by Zhu et al., (2009b) obtaining a 48h-LC_{50} value for ZnO-NPs of 1.48 $mg.L^{-1}$ for the mortality endpoint. For the other endpoints,

concentration-dependent doses were observed. Possible, the reduction of food uptake, may have in turn affected growth and reproduction of *D. magna*.

2.2.5.3. Toxicity to fish

Studies investigating effects *in vivo* of ZnO-NPs in fish are still scarse (Zhu et al., 2008; Zhu et al., 2009a; Xiong et al., 2011; Yu et al., 2011) and they have been especially focused on the toxicity effects at early developmental stages (Yu et al., 2011) (Table 1.2).

Zhu et al., (2008) studied the effects of ZnO-NPs (20 nm) to zebrafish (*Danio rerio*) in several endpoints such as embryo survival and hatching rate and after compared with ZnO micro-sized particles (1µm).

Embryo survival and hatching rate of zebrafish showed a dose-dependency with the increase of ZnO-NPs concentrations. However, no statistical differences were found between Zn compounds for both endpoints, recording a 96h-LC_{50} value of 1.793 $mg.L^{-1}$ for ZnO-NPs and 1.550 $mg.L^{-1}$ for ZnO bulk for embryo survival and a 84h-EC_{50} value of 2.065 $mg.L^{-1}$ for ZnO-NPs and 2.066 $mg.L.^{-1}$ for ZnO bulk for hatching rate.

Dissolution of Zn^{2+} ions from ZnO-NPs and ZnO bulk were assessed so that toxicological tests were performed again for the same endpoints; results showed that Zn^{2+} concentrations affected the survival rates in 86.7% at the end of 96h and 90% of the hatching rates at the end of 84h.

It was then clear that the release of zinc ions contributed for the toxic effects in zebrafish development. However, authors reported that toxicity of Zn^{2+} was significantly lower that the toxicity of ZnO-NPs, therefore suggesting that maybe there are others factors responsible for the toxic effects, such as ROS production.

The ecotoxicological tests developed by these authors were performed in Mili-Q water. This fact makes us raise the question if zebrafish embros could survive during 96h only in Mili-Q water.

Later, reported by the same author, Zhu et al.,(2009a) assessed the influence of micro-sized ZnO-NPs during 96h to the embryonic development of zebrafish (*Danio rerio*), reporting a 84h-EC_{50} value (for embryo hatching) of 23.06 $mg.L^{-1}$ (Table 1.2).

In this study they also measured the release of Zn^{2+} ion concentrations of all test solutions, by GFAA, to determine whether zinc played an important role to the development of *D. rerio*. By exposing zebrafish embryos to the concentrations of Zn^{2+}

released from ZnO-NPs, it was observed that zinc ions did not cause apparent toxicity to zebrafish when compared with the same concentrations of ZnO-NPs, which again implied that Zn^{2+} could not have been the only toxic agent to zebrafish.

Yu et al., (2011) (Table 1.2.) studied the effects of ZnO-NPs (30 nm) in adult zebrafish and compared the effects of ZnO-NPs with their bulk counterpart (500 nm) and Zn^{2+} ions, in the form of $ZnSO_4$. The 96h-LC_{50} value obtained for ZnO-NPs was 3.70 $mg.L^{-1}$. ZnO bulk suspensions showed to be more toxic than ZnO nanoparticles, however not statistically different, with a 96h-LC_{50} value of 2.53 $mg.L^{-1}$, probably due to aggregation of ZnO-NPs suspensions as reported by authors. For Zn^{2+}, the toxicity to zebrafish showed to be higher when compared with the ZnO particles (96h-LC_{50} = 7.480 $mg.L^{-1}$).
Therefore authors suggested that dissolved zinc from ZnO suspensions may have contributed for the acute toxicity to zebrafish and that the mechanisms of toxicity of Zn^{2+} may be different from ZnO particles. OH^- generation was also determined but it was concluded they played a small role in the toxicity to zebrafish since ZnO-NPs and ZnO bulk presented very different abilities to generate OH^- thus not being the main factor for toxicity.

Xiong et al., (2011) recently reported dose-dependency toxicity for ZnO-NPs (30 nm), ZnO bulk (500 nm) and Zn^{2+} (in the form of $ZnSO_4$) in adult zebrafish. 96h-LC_{50} values for all Zn compounds obtained were 4.26 $mg.L^{-1}$ to ZnO-NPs and 3.31 $mg.L^{-1}$ to ZnO bulk and 8.06 $mg.L^{-1}$ to Zn^{2+} ions, with statistical differences between ZnO-NPs and Zn^{2+} and between ZnO bulk and Zn^{2+} (Table 1.2).
Like the previous study and given the results, it was suggested that the dissolution of zinc ions it may contribute for the toxicity of ZnO-NPs and ZnO bulk but it was not considered as the main lethal mechanism for ZnO suspensions.

2.2.5.4. Toxicity to other organisms

There are also other studies concerning the effects of ZnO-NPs to organisms such as bacteria, amphibians and plants but at a lower scale.

Due to its antimicrobial activity, studies on the effects of ZnO-NPs to different bacteria species have already been documented.

Jiang at al., (2009) reported that ZnO-NPs (20 nm) were the most toxic to three bacteria species (*Bacillus subtilis, Escherichia coli and Pseudomonas fluorescens*) in a set of three different NPs studied (ZnO, SiO_2 and Al_2O_3).
This toxicity was compared with the respective counterparts. ZnO bulk showed no or lower toxicity, indicating that particle size was responsible for a difference in the toxicity of both Zn compounds. Authors also concluded that toxicity of ZnO-NPs was not only due to the dissolution of Zn^{2+} because the three species of bacteria showed different sensitivities when exposed to concentrations of 2 $mg.Zn^{2+}.L^{-1}$.

In a more recent study, Kumar et al., (2011b) showed that concentrations as low as 8 $\mu g.L^{-1}$, ZnO-NPs were able to significantly reduce cell viability and membrane permeability of *E. coli*. During the exposure of ZnO-NPs, molecular perturbations induce by these metal oxide nanoparticles were observed (ROS production, glutathione depletion, increased lipid peroxidation, LDH release and DNA damage) which may have caused cell death in *E. coli*.

These results come to demonstrate why ZnO-NPs are often referred as antibacterial agents with potential health-related applications (Jones et al., 2008; Liu et al., 2009).

Regarding amphibians, a recent study showed the effects of ZnO-NPs (40-100 nm) in the development of a frog specie, *Xenopus laevis* (Nations et al., 2011). The results showed that exposures of 2 $mg.L^{-1}$ significantly increased mortality and did not allow the organisms to complete metamorphosis (Nations et al., 2011). On the other hand, concentrations lower than 0.125 $mg.L^{-1}$ induced faster development in *X. laevis*.
Knowing that Zn is an essential metal for many cell processes (WHO, 2001), this confirms that at low doses, Zn can stimulate growth and metamorphosis of *X. laevis* and as expected its increase above a certain threshold level caused adverse effects.

Taking into account all these studies mentioned, it is noticeable that toxic effects are highly dependent on the target organism, which means that different groups of organisms show different responses towards nanoscale ZnO (Barrena et al., 2009; Wong et al., 2010). Therefore, to better understand the potential toxic effects of ZnO-NPs and other NPs, toxicity testing should be performed in a wide range of organisms of the food chain (Barrena et al., 2009).

Although many studies have investigated the toxicity of ZnO-NPs to aquatic organisms, data on marine species are particularly lacking (Wong et al., 2010). Investigations on marine species should be considered because the physicochemical properties and behaviour of NPs may change due to the characteristics (e.g., ionic strength, pH, NOM) of the environment where they are released (Wong et al., 2010).

It is also known that dissolution rate plays a major role in the toxicity of NPs (Miao et al., 2010).

Even if there are already available in literature studies concerning the dynamics of solubility of ZnO-NPs and to what extent this process affects toxicity, it is still not yet fully understood (Miao et al., 2010). In most of the cases involving ZnO-NPs the toxic effects are attributed to dissolved Zn ions from ZnO-NPs dispersions and not to nano ZnO itself (Wiench et al., 2009; Mortimer et al., 2010; Wong et al., 2010; Peng et al., 2011).

For instance, Franklin et al., (2007) using a physical method, i.e. dialysis membrane with a pore size of about 1000 Da molecular weight cutoff, which is permeable only to Zn ions showed that both nano and bulk ZnO suspensions yielded similar dissolved Zn concentrations resulting in similar toxicities.

However, Zhu et al., (2009a) speculated that Zn^{2+} by itself could not be the only cause of toxicity but a combined effect of both Zn^{2+} and nanoscale ZnO.

Table 1.2. Overview of the toxicity of ZnO nanoparticles to aquatic organisms.

Test Organism	Particle size (nm)	Exposure Media	Concentrations range	Endpoint	Test Duration	Observations	Reference
Thalassiosia pseudonana	20	*f2-Si medium*	-	Growth inhibiton	96h	IC_{50} = 4.56 mg.L^{-1}	(Wong et al., 2010)
Thalassiosira pseudonana	20-30	*f/2 medium*	0,10,100,500,1000 µg.L^{-1}	Growth Inhibition	96h	IC_{50} value not shown	(Miller et al., 2010)
Skeletonema costatum	20	*f2-Si medium*	-	Growth inhibiton	96h	IC_{50} = 2.36 mg.L^{-1}	(Wong et al., 2010)
Skeletonema marinoi	20-30	*f/2 medium*	0, 10, 100, 500, 1000 µg.L^{-1}	Growth Inhibition	96h	IC_{50} value not shown	(Miller et al., 2010)
Dunaliella tertrolecta	20-30	*f/2 medium*	0, 10, 100, 500, 1000 µg.L^{-1}	Growth Inhibition	96h	IC_{50} value not shown	(Miller et al., 2010)
Isochrysis galbana	20-30	f/2 medium	0, 10, 100, 500, 1000 µg.L^{-1}	Growth Inhibition	96h	IC_{50} value not shown	(Miller et al., 2010)
Pseudokirchneriella subcapitata	30	U.S. EPA medium	25 – 600 µg Zn.L^{-1}	Growth Inhibition	72h	nZnO-IC_{50} = 68 µg.L^{-1} Bulk-IC_{50} = 63 µg.L^{-1} $ZnCl_2$-IC_{50} = 61 µg.L^{-1}	(Franklin et al., 2007)
Pseudokirchneriella subcapitata	50-70	algal growth medium(OECD, 1984)	0.1-0.5 mgZn/l	Growth Inhibition	72h	nZnO-IC_{50} = 0,042 mg.L^{-1}; Bulk-IC_{50} = 0,037mg.L^{-1}; $ZnSO_4$-IC_{50} = 0,042mg.L^{-1}	(Aruoja et al., 2009)
Chlorella sp.	20 ± 5	SE media	0, 5, 10, 20, 50, 100, 200, 1000 mg.L^{-1}	Growth Inhibition	6 days	nZnO-EC_{30} = 20 mg.L^{-1} Bulk-EC_{30} = 100 mg.L^{-1} Zn^{2+}-EC_{30} = 2mg.L^{-1}	(Ji et al., (*in press*))
Daphnia magna	20	Reconstitured Water	0.01 – 5 mg.L^{-1}	Immobilisation	48h	nZnO-EC_{50} = 0.622 mg.L^{-1} nZnO-LC_{50} =1.511 mg.L^{-1} Bulk-EC_{50} = 0.481 mg.L^{-1} Bulk-LC_{50} = 1.250 mg.L^{-1}	(Zhu et al., 2009b)
Daphnia magna	50-70	Synthetic Freshwater	0.01 – 10000 mg.L^{-1}	Immobilisation	48h	nZnO-LC_{50} = 3.2 mg.L^{-1} Bulk-LC_{50} = 8.8 mg.L^{-1} $ZnSO_4$-LC_{50} = 6.1 mg.L^{-1}	(Heinlaan et al., 2008)

Table 1.2. (*Continued)*

Daphnia magna	50-70	-	0.02 – 5 $mg.L^{-1}$	Immobilisation	48h	LC_{50} = 2.1 ± 0.1 $mg.L^{-1}$	(Naddafi et al., 2011)
Daphnia magna	70	Artificial water	0.001 – 10 $mg.L^{-1}$	Immobilisation	48h	EC_{50} = 2.6 ± 1.04 $mg.L^{-1}$	(Blinova et al., 2010)
Daphnia magna	< 200	Artificial Elendt M4 medium	0.1 – 100 $mg.L^{-1}$	Immobilisation	48h	EC_{50} = 7.5mg L^{-1}; EC_{10} = 5.2 $mg.L^{-1}$	(Wiench et al., 2009)
Ceriodaphnia affinis	15 – 350	Reconstitured Water	0.02 – 200 $mg.L^{-1}$	Immobilisation	24h	LC_{50} = 0.09 ± 0.001 $mg.L^{-1}$	(Tomilina et al., 2011)
Thamnocephalus platyurus	50-70	Synthetic Freshwater	0.01 – 10000 $mg.L^{-1}$	Mortality	24h	nZnO-EC_{50} = 0.18 $mg.L^{-1}$ Bulk-EC_{50} = 0.24 $mg.L^{-1}$ $ZnSO_4$-EC_{50} = 0.98 $mg.L^{-1}$	(Heinlaan et al., 2008)
Thamnocephalus platyurus	70	Artificial water	0.001 – 10000 $mg.L^{-1}$	Mortality	24h	LC_{50} = 0.14 $mg.L^{-1}$ ± 0.02	(Blinova et al., 2010)
Tigriopus japonicus,	20	natural seawater	-	Mortality	96h	LC_{50} = 0.85 $mg.L^{-1}$	(Wong et al., 2010)
Elasmopus rapax	20	Natural seawater	-	Mortality	96h	LC_{50} = 1.19 $mg.L^{-1}$	(Wong et al., 2010)
Danio rerio	20	Mili-Q water	0, 0.1, 0.5, 1, 5,10,50 $mg.L^{-1}$	Embryo survival(ES) and hatching rate(HR)	96h	nZnO-$LC_{50(ES)}$ = 1.793 $mg.L^{-1}$ Bulk-$LC_{50(ES)}$ = 1.550 $mg.L^{-1}$ nZnO-$LC_{50(HR)}$ = 2.065 $mg.L^{-1}$ Bulk-$LC_{50(HR)}$ = 2.066 $mg.L^{-1}$	(Zhu et al., 2008)
Danio rerio	20	-	0.1, 0.5, 1, 5, 10, 50, 100$mg.L^{-1}$	Embryo/larvae survival and embryo hatching rate	96h	84h-EC_{50} (embryo hatching) = 23.06 $mg.L^{-1}$	(Zhu et al., 2009a)
Danio rerio	20-70	Fresh water	0, 2, 5, 10, 30 and 50 $mg.L^{-1}$	Mortality	96h	nZnO-LC_{50} = 4.92 $mg.L^{-1}$ Bulk-LC_{50} = 3.31 $mg.L^{-1}$ $ZnSO_4$-LC_{50} = 8.06 $mg.L^{-1}$	(Xiong et al., 2011)
Danio rerio	30	Distilled water	1, 2, 5,10,30,50 $mg.L^{-1}$	Mortality	96h	nZnO-EC_{50} = 3.969 $mg.L^{-1}$ Bulk-EC_{50} = 2.525 mg/L^{-1} $ZnSO_4$-EC_{50} = 7.480 $mg.L^{-1}$	(Yu et al., 2011)

2.3. Overview and conclusions

The effect of ZnO-NPs often present non consensual results since there are authors suggesting that the toxicity associated to ZnO-NPs comes from dissolved ions whether others suggest that NPs are the mainly precursors of toxicity.

There are also studies indicating that giving the fact ZnO-NPs present smaller particle sizes thus greater ability to penetrate into organisms they are able cause higher toxicity when compared with the same material with higher particle size. Concerning this topic, there is not also a consensual opinion because some studies report similar toxicities between ZnO-NPs and the respective bulk counterpart.

Many authors have been reporting during the previous years, concentrations of ZnO-NPs that produce toxic effects, especially in the species most used in regulatory testing. However, this kind of studies should include a larger number of organisms since there is still some lack of information on several groups of organisms as reported previously.

Most of these studies rely only to short-exposure times whereas very few studies have investigated chronic exposures in organisms. Chronic toxicity tests would be closer to realistic conditions since NPs are likely to persist when they are released into the environment and can be subject of dissolution, aggregation processes and interactions with other pollutants that in turn can cause influence their toxicity.

Despite the great developments already made in the multidisciplinary field of nanotechnology during the past years, there are still some gaps that need to be addressed in order to allow a better comprehension of the real impacts of ENPs to the environment and human health.

For instance, chemical characterization of ENPs in aquatic test media should be performed for the development of ecotoxicological tests. These approaches using the knowledge on physico-chemistry characteristics of NPs is crucial to predict their behaviour in water and consequent effects to aquatic organisms since many of the conflicting results reported may be associated with differences of size distributions among the ENPs used, the chemical composition, surface structure, shape and others, possibly affecting the behaviour and fate of NPs. The methods by which ENPs are synthesized, the test media for exposure and the protocol adopted may also play a role on the behaviour of these nanoscale materials.

Another necessity to aid on the development of studies regarding the toxicity of ENPs to the aquatic environment is the development of techniques to predict environmental concentrations (PECs) of nanoparticles. This is an important aspect that needs to be considered since they may help to identify possible adverse effects in aquatic ecosystems. Therefore these techniques could allow the creation of secure measures before the release of ENPs into the environment.

Even if the emergence of nanotechnology has brought us significant advantages it has also brought us great concerns since more and more ENPs are being released in a big scale into the environment (nanowaste). This is expected to increase in the upcoming years without having information on how ENPs should be treated and disposed after their use in consumer products since nanoparticles do not behave in the same manner as the normal waste, requiring specific legislations.

Although there is currently many toxicological data available regarding the impacts of ENPs to the environment, more efforts from the scientific community are still necessary in an attempt to promote reliable (nano)ecotoxicological-based science.

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Chapter 3

Effect of zinc oxide nanoparticles in *Daphnia magna*: size dependent effects and counterparts

3. Effect of zinc oxide nanoparticles in *Daphnia magna*: size dependent effects and counterparts.

3.1. Abstract

As the production of zinc oxide nanoparticles (ZnO-NPs) and other metal oxides is exponentially increasing, it is important to investigate potential environmental and health impacts of such nanoparticles.

Particle size is an important parameter to be considered when analysing the toxicity of nanoparticles (NPs), since it is known that at the nanosize range the properties of materials differ substantially from the respective bulk counterpart.

Therefore, an aquatic model organism, *Daphnia magna*, was used to investigate the effect of ZnO-NPs with two different particle size (30 nm and 80-100 nm) and then compare them with ZnO-micro-sized and ionic counterparts ($ZnCl_2$) on immobilization, feeding inhibition and reproduction endpoints.

The 48h-LC_{50} values for immobilization ranged between 0.76 mg.Zn^{2+}.L^{-1} for Zn (in the form of $ZnCl_2$) and 1.10 mg.Zn^{2+}.L^{-1} for ZnO-NPs 80-100 nm. For the chronic exposures (reproduction and feeding inhibition tests), D. *magna* showed a reduction in the offspring production and feeding rate activities when exposed to high concentrations of all zinc compounds.

Keywords: Zinc oxide nanoparticles, *Daphnia magna*, particle size, toxicity

3.2. Introduction

Metal oxide nanoparticles (e.g., ZnO, TiO_2) (Nowack and Bucheli, 2007) as well as fullerenes (e.g., C_{60}) and carbon nanotubes (CNT) are the most studied nanoparticles (NPs) (Baun et al., 2008) in ecotoxicology as a consequence of the fast increase on the use of these nanoscale materials for novel technologies. However, the development of these nanotechnologies is still ahead of their inherent risk evaluation regarding the impacts of NPs to the environment (Krysanov et al., 2010) since it's not yet certain if NPs are inherently toxic due to contradictory results found in the literature (Dybowska et al., 2011).

As a consequence of the extensive use/production of NPs, they are likely to be released into the environment, most especially into the aquatic environment, through bathing recreation (Wiench et al., 2009), run-off and wastewater from domestic/industrial applications (Baun et al., 2008). Wong et al., (2010) reported that the discharge rate into the marine environment of nano metal oxides used in personal care products (e.g., sunscreens) runs at about 25% of the total amount of product applied on the skin. These occurrences will lead inevitably to some degree of environmental and human exposure, instigating the need to catch up the fast growth of such multidisciplinary field (Elsaesser and Howard, 2011).

Zinc oxide nanoparticles (ZnO-NPs) have received much attention because of its low production costs and features such as large volume to area ratio, long life cycle (Hong et al., 2009) and high UV radiation absorption (Handy et al., 2008). They have been applied in a variety of personal care products such as sunscreens, toothpastes and cosmetics (Blinova et al., 2010; Xiong et al., 2011). Due to the versatility of these nanoparticles, they can also be applied in coatings, paints (Blinova et al., 2010), for environmental remediation processes (Xiong et al., 2011), wastewater treatments (Wong et al., 2010), textiles (Mortimer et al., 2010), ceramics, rubber manufacture, food additives, biosensors (Brayner et al., 2010), in catalysts, batteries, (Bystrzejewska-Piotrowska et al., 2009) and as an antibacterial agent (Zhang et al., 2007).

It has been reported that when bulk counterparts are made into smaller particles, their physicochemical features change increasing their surface reactivity (Elsaesser and Howard, 2011) thus being able to interact/penetrate more efficiently with/into organisms (Brayner et al., 2010; Xiong et al., 2011) possibly trigging adverse responses.

For ZnO-NPs it already exists a considerable literature reporting toxic effects in several organisms, such as bacteria (Jiang et al., 2009; Baek and An, 2011; Premanathan et al., 2011), algae (Franklin et al., 2007; Aruoja et al., 2009; Ji et al., (*in press*)) and fish (Zhu et al., 2009a; Yu et al., 2011). However, most of the studies performed in aquatic organisms use aquatic invertebrates such as the cladoceran *Daphnia magna* as the standard organism (Heinlaan et al., 2008; Wiench et al., 2009).

Daphnia magna is a freshwater invertebrate that has been used extensively for the past 20 years in regulatory testing and ecotoxicological research and it presents features that makes them suitable for laboratory testing (Sanna, 1995). Among them are the small size, high fecundity, short life cycle, reproduction by parthenogenesis, ubiquitous occurrence and ease to handle in laboratory (Sanna, 1995).
Due to the high sensitivity to environmental pollutants (Wang and Guan, 2010) and representative of the food-web chain because they act as food and energy link between primary producers and secondary consumers, cladocerans can be considered a good toxicity model organism to predict the toxicity of pollutants to ecosystems (Heinlaan et al., 2008).
But considering the new features that NPs bring to science, already standardized protocols have sometimes to be adapted and revised in order to evaluate accurately the effects of NPs to the biota.

Therefore, the aim of this study was to evaluate the related size-effects of ZnO-NPs considering two different sizes (30 nm and 80-100 nm) and also to compare with the effects of ZnO micro-sized (200 nm) and ionic counterparts ($ZnCl_2$) in D. *magna.* For that effect immobilisation, feeding inhibition and reproduction were evaluated.

3.3. Material and Methods

Chemicals

ZnO nanoparticles (30 nm and 80-100 nm) and the bulk form of ZnO (200 nm) were supplied by Nanotrade/Microniser as powder.

Zinc Chloride (CAS no.7647-85-7, 98% purity) with a molar mass of 136.40 $g.mol^{-1}$ was purchased by Riedel-de Haën.

Preparation of suspensions

Zinc oxide stock dispersions (both nano and bulk) of 50 $mg.L^{-1}$ were prepared in Mili-Q water. All suspensions were prepared by sonication for about 30 minutes. Test suspensions were immediately prepared prior to test (immobilisation and feeding inhibition) or for media renewal for the reproduction tests.

A 20 $mg.L^{-1}$ stock solution was prepared for zinc chloride in ASTM and shaken vigorously after. For the reproduction tests, since it was necessary to renew the media every two days, the solution was stored at 4ºC during the test duration.

Nanoparticles characterization

The size distribution of ZnO-NPs and respective bulk dispersions was determined by transmission electron microscopy (TEM) in distilled water.

Test organism and culture maintenance

The freshwater crustacean *D. magna* Strauss, clone K6, was used as the standard test organism. Daphnids were maintained in aquariums of 3 L with reconstituted hard water (ASTM, 1980) and kept at a constant temperature of 20±1ºC with a 16:8h light:dark photoperiod. Culture medium was renewed 3 times a week. Daphnids were fed with *Pseudokirchneriella subcapitata* at a concentration of $3x10^5$ $cell.mL^{-1}$ and with 6 $ml.L^{-1}$ of a seaweed extract. Only the neonates from third to fifth brood were used in toxicity tests to minimize variability. Neonates from sixth brood were used to replace old cultures. Additionally, to verify organism sensitivity, acute tests with the reference compound potassium dichromate were performed at least twice a year.

Acute toxicity tests

Immobilization tests were performed in accordance with the OECD guideline 202 (OECD, 2004). The tests were performed with five replicates for each concentration test plus a control. The daphnids were not fed during the setup. The tests were conducted at constant temperature of 20±1ºC with a 16:8h light:dark photoperiod.

Five neonates (<24hours) randomly chosen were placed in 50 ml glass beakers per replicate to a range of concentrations during the experimental setup.

After 24h and 48h, the immobilisation (inability to swim after a gentle agitation of the glass beaker) and mortality were recorded and the LC_{50} values calculated.

The nominal concentrations for ZnO-NPs (both nano and bulk) ranged between 0.25 – 10 $mg.L^{-1}$. For zinc chloride the nominal concentrations ranged between 0.4 – 13.3 $mg.L^{-1}$. Taking into account the nominal concentrations of zinc for all the ZnO-NPs and ZnO micro-sized and $ZnCl_2$ they ranged between 0.2 – 8 $mg.Zn^{2+}.L^{-1}$ and 0.2 – 6.4 $mg.Zn^{2+}.L^{-1}$, respectively. The results are expressed in values of $mg.Zn^{2+}.L^{-1}$ in order to compare solely zinc toxicity effects.

Feeding Inhibition tests

Neonates (<24h old) were separated from the main culture to other aquarium until they reach 4 to 5 days old, being equivalent to the fourth instars. This life stage is adequate for this experimental setup because it allows to perform feeding inhibition bioassays in a single molt cycle, avoiding molting interference in the feeding rates of daphnids as observed by McWilliam and Baird (2002).

For each concentration test, plus a control, five replicates with five individuals per treatment were used. The five neonates were randomly chosen and placed in 170 ml glass beakers with 100 ml of the test substance and fed for 24h with algae P. *subcapitata* at a concentration of $5x10^5$ $cell.mL^{-1}$. To establish the initial algal concentrations, a blank set of 3 replicates (with no daphnids) were added to the experimental setup.

All the glass beakers were kept in the dark to ensure uniform feeding rates during 24h (*exposure period*) and lack of algae growth. After this exposure time, daphnids were transferred into 50 ml glass beakers containing clean ASTM with food also at a concentration of $5x10^5$ $cells.mL^{-1}$ and allowed to feed for 4h (*post-exposure*) in the dark.
At the end of both periods (*exposure and post-exposure period*), each replicate was vigorously shaken to resuspend cells and the absorbance values were determined at 440 nm by spectrophotometry (Jenway 6505 Spectrophotometer UV-VIS). Individual feeding rates for 24h exposure and 4h post-exposure were determined according by Allen et al. (1995).

Nominal test concentrations for ZnO 30 nm, ZnO 80-100 nm and ZnO 200 nm ranged from 1.2 – 2.4 $mg.L^{-1}$, 2 – 4 $mg.L^{-1}$ and 0.5 – 3 $mg.L^{-1}$, respectively. For $ZnCl_2$ the nominal test concentrations ranged from 0.8 – 6.7 $mg.L^{-1}$. In terms of Zn, the nominal concentrations for ZnO-NPs and bulk ranged from 0.4 – 3.2 $mg.Zn^{2+}.L^{-1}$ and for $ZnCl_2$ from 0.4 – 12.8 $mg.Zn^{2+}.L^{-1}$. Data are expressed as $mg.Zn^{2+}.L^{-1}$.

Chronic toxicity tests

21 days chronic tests were conducted according to OECD 211 guideline (OECD, 1998). For each treatment and control 10 replicates of 1 individual each (<24h old) were used. The exposure was conducted in 50 ml glass beakers containing *P. subcapitata* at a concentration of $3x10^5$ cells.mL^{-1} and seaweed extract and kept at constant temperature of 20±1ºC with a 16:8h light:dark photoperiod.

The renewal of the test medium for all treatments and control was carried out every 2 days. Additionally, the individuals were fed daily during the test period.
Immobilisation and/or mortality of the parent daphnids and offspring were assessed daily. The live neonates were counted and removed from the test glass beakers after appearance of a brood.

Test-medium parameters (pH, dissolved oxygen and conductivity) were measured at the beginning, middle and end of each test in both old and renewed medium.

Daphnids were measured at the beginning of the test and also at the end to assess the differences of size between the control and the test concentrations.

Nominal concentrations used for ZnO 30 nm, ZnO 80-100 nm and ZnO 200 nm ranged between 0.125 – 0.75 $mg.L^{-1}$, 0.125 – 0.75 $mg.L^{-1}$ and 0.0375 – 0.45 $mg.L^{-1}$, respectively. For zinc chloride the nominal concentrations ranged from 0.3 – 0.8 $mg.L^{-1}$. In terms of Zn, the nominal concentrations for ZnO-NPs and bulk ranged from 0.03 – 0.6 $mg.Zn^{2+}.L^{-1}$ and for $ZnCl_2$ zinc nominal concentrations ranged from 0.15 – 0.40 $mg.Zn.L^{-1}$. Data are expressed as $mg.Zn^{2+}.L^{-1}$.

Statistical analysis

The 48-hrs LC_{50} values for immobilisation of *D. magna* exposures were calculated by the SigmaPlot software using a nonlinear regression with a sigmoidal function that showed the better adjustment (Systat, 2004).
The 24h-EC_{50} and 4h-EC_{50} values for feeding activity and 21d-EC_{50} values for reproduction were calculated using a nonlinear regression by a logistic 3-parameter equation or 4-parameter equations (Systat, 2004).

To determine statistical differences between control data and exposure treatment data were analysed by a one-way ANOVA, followed by the Dunnett's test when appropriated. For data that failed the normality testing, a non-parametric Kruskal-Wallis test was performed followed by Dunn's method to access multiple comparisons between

treatments and control (Systat, 2004). All significant differences were established at $p<0.05$.

3.4. Results

Particle characterization of ZnO-NPs and respective bulk material

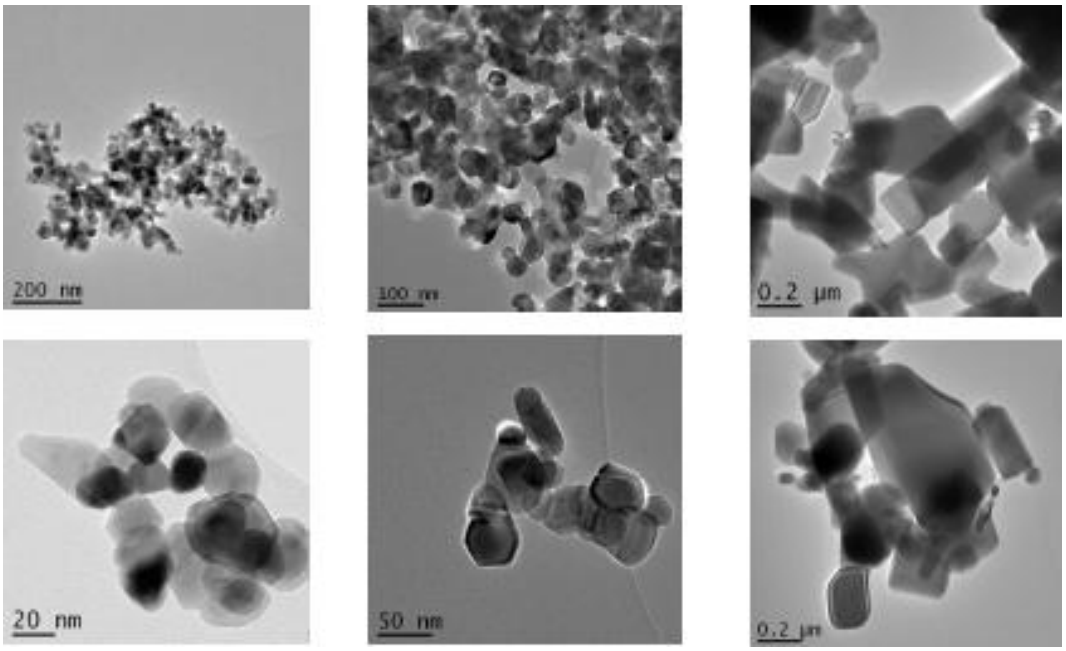

Fig. 2.1. Transmission electron microscope images (TEM) of zinc oxide nanoparticles of 30 nm (*left*), 80-100 nm (*center*) and > 200 nm (*right*) in distilled water.

TEM images are shown in Fig. 2.1. For the ZnO-NPs of 30 nm it was observed an average primary particle size of approx. 30-50 nm, mostly in aggregates from 100 nm to micron sized. TEM images for ZnO-NPs 80-100 nm showed primary particle sizes of approx. 30-80 nm in which very few separate particles where observed, being found mostly in aggregates from a few particles to micron sized. For ZnO micro-sized TEM images detected primary particle size of approx. 50-500 nm with high number of high aspect ratio particles mainly in large aggregates.

Acute toxicity of different sized ZnO NPs, ZnO micro-sized and $ZnCl_2$ to Daphnia magna

The 48h-LC_{50} values for the ZnO forms and $ZnCl_2$ are shown in Table 2.1. Their acute toxicity increased with higher concentrations, demonstrating a dose dependency. After 48h of exposure, 100% of mortality was observed for the two highest concentrations of ZnO-NPs 80-100 nm and for the three highest concentrations of ZnO-NPs of 30 nm, ZnO micro-sized and $ZnCl_2$.

Zinc chloride, showed higher toxicity to *D. magna* when compared with ZnO NPs and micro-sized, with a 48h-LC_{50} of 0.76 mg.Zn^{2+}.L^{-1}. ZnO forms showed similar toxicities.

Exposure and post-exposure of feeding inhibition tests

Feeding activities, measure as feeding rates (cell/ml), showed in general decrease of values with increasing concentrations in all ZnO-NPs, ZnO micro-sized and $ZnCl_2$ on both exposure (24h) and post-exposure (4h) experiments (Fig. 2.2.).

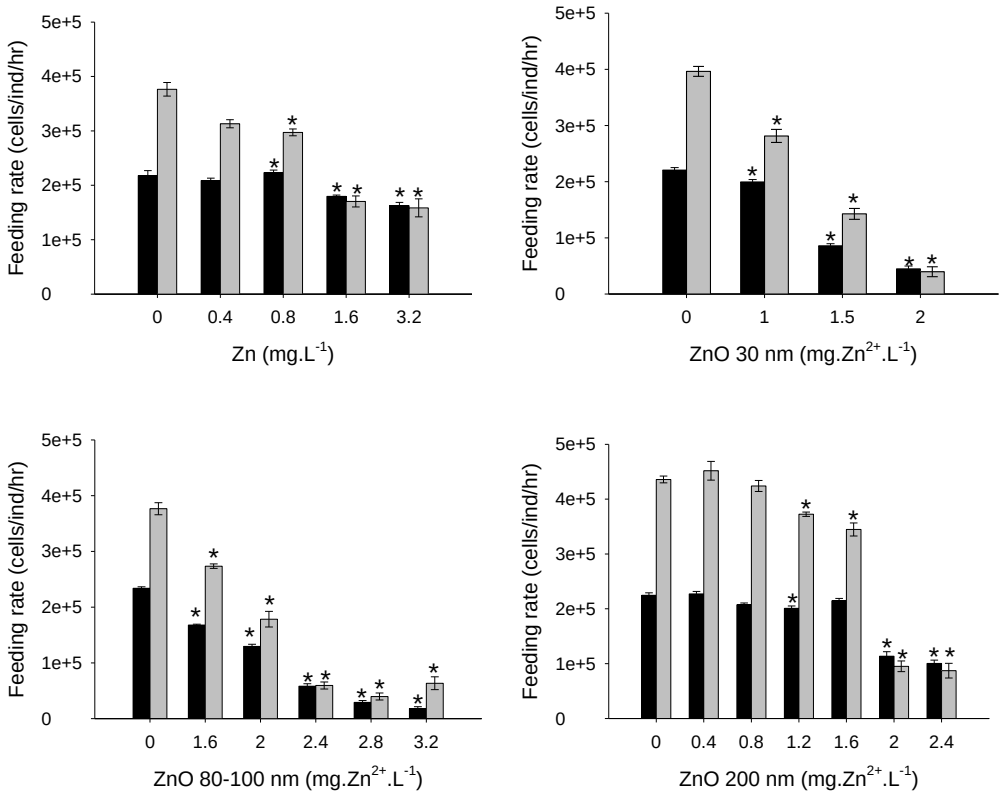

Fig. 2.2. Feeding rates of *D. magna* during 24h of exposure (black bars) and 4h of post-exposure (grey bars) at concentrations of Zn, ZnO-NPs (30 and 80-100 nm) and ZnO micro-sized. Data is expressed as mean values ± standard error. (*) Statistical differences at $p<0.05$.

No mortality was observed during the 24h of exposure in the tests. Statistical differences were observed between controls and treatments for the two ZnO-NPs, 30 and 80-100 nm, (one way ANOVA, $F_{3,19}$=386.17, p<0.001, Dunnett's method, p<0.05; one way ANOVA, $F_{5,28}$=651.45, p<0.001, Dunnett's method, p<0.05), ZnO micro-sized (one way ANOVA, $F_{6,33}$=101.92, p<0.001, Dunnett's method, p<0.05) for 24h of exposure.

ZnO-NPs of 30 nm showed to be more toxic than the ZnO-NPs of 80-100 nm and ZnO micro-sized with an EC_{50} value of 1.41 mg.Zn^{2+}.L^{-1} and a LOEC of 1 mg.Zn^{2+}.L^{-1}(Table 2.1).

For $ZnCl_2$ it was also observed statistical differences between control and treatments (one way ANOVA, $F_{4,24}$=20.19, p<0.001; Dunnett's method, p<0.05). Mortality on the two highest concentrations used (6.4 and 12.8 mg.Zn^{2+}.L^{-1}) was observed, so they were not used for the statistical analysis. The effect concentration that reduced 50% the feeding rates could not be calculated because the highest concentration used where no mortality occurred showed less than 50% of feeding inhibition (EC_{50}> 3.2 mg.Zn^{2+}.L^{-1}).
Post-exposure experiments showed a decrease in the feeding activity with significant difference at 0.8, 1.6 and 3.2 mg.Zn^{2+}.L^{-1} for $ZnCl_2$ (Dunnett's method, p<0.05), 1, 1.5 and 2 mg.Zn^{2+}.L^{-1} for ZnO-NPs of 30 nm (Dunnett's method, p<0.05), 1.6, 2, 2.4, 2.8 and 3.2 mg.Zn^{2+}.L^{-1} for ZnO-NPs of 80-100 nm (Dunnett's method, p<0.05) and 1.2, 1.6, 2 and 2.4 mg.Zn^{2+}.L^{-1} for ZnO micro-sized (Dunnett's method, p<0.05).

Chronic toxicity of different sized NPs, bulk counterparts and $ZnCl_2$ to Daphnia magna

Mortality of parent control animals at the end of all chronic tests was always ≤20%. Additionally, the mean number of live offspring produced per parent control daphnids ranged between 149 and 121 (data not shown), thus making all tests valid according to the OECD guideline 211 (OECD, 1998).

In all tests performed, pH ranged between 7.52 and 8.50, dissolved oxygen between 8.47 and 12.63 mg.L^{-1} and conductivity between 451 and 615µS/cm going in accordance with the validation criteria of the protocol adopted.

The reproduction response of D. *magna* was affected at low concentrations tested of $ZnCl_2$, ZnO NPs and ZnO micro-sized (Fig. 2.3.).
The last nominal concentration for ZnO-NPs 30 nm (0.6 mg.Zn^{2+}.L^{-1}) was not used in the data analysis due to the high mortality rate occurred during the last week of the test.

The total live offspring per surviving female for $ZnCl_2$ (one way ANOVA, $F_{3,25}$ = 81.71, $p \leq 0.001$, Dunnett's method, $p<0.05$), ZnO-NPs of 30 nm (one way ANOVA, $F_{3,38}$ = 87.36, $p \leq 0.001$, Dunnett's method, $p<0.05$, after square transformation), ZnO-NPs of 80-100 nm (one way ANOVA, $F_{4,45}$ = 46.82, $p \leq 0.001$, Dunnett's method, $p<0.05$) and ZnO micro-sized (Kruskal-Wallis one way ANOVA, H=30.08, df=5, $p \leq 0.001$, Dunn's method, $p<0.05$) showed significant differences when compared with the respective control.

The LOEC values for ZnO-NPs 30 nm, ZnO-NPs 80-100nm, ZnO micro-sized and $ZnCl_2$ caused an inhibition rate in reproduction of ~ 29 %, 31 %, 62 % and 24%, respectively.

Table 2.1. Summary of the effects of all test compounds on immobilization, feeding activities and reproduction of *Daphnia magna*. Results are expressed as mean ± standard error; R^2 is the coefficient of determination; NOEC is defined as No-observed effect concentration; LOEC is defined as Lowest observed effect concentration. Data are expressed as $mg.Zn^{2+}.L^{-1}$.

	Acute toxicity tests		**Feeding inhibition tests**								**Chronic toxicity tests**			
Substance	**48h-LC_{50}**	**R^2**	**24h-EC_{50}**	**R^2**	**NOEC**	**LOEC**	**4h-EC_{50}**	**R^2**	**NOEC**	**LOEC**	**21d-EC_{50}**	**R^2**	**NOEC**	**LOEC**
$ZnCl_2$	0.76	1	>3.2 <12.8	-	0.8	1.6	1.92 ± 0.25	0.871	-	0.4	>0.25 <0.40	-	-	0.15
ZnO 30 nm	1.02 ± 0.24	0.998	1.41 ± 0.03	0.972	-	1	1.27 ± 0.03	0.975	-	1	0.26 ± 0.03	0.833	-	0.1
ZnO 80-100 nm	1.10 ± 0.05	0.998	2.00 ± 0.03	0.984	-	1.6	1.91 ± 0.05	0.951	-	1.6	0.36 ± 0.03	0.799	0.1	0.2
ZnO > 200nm	0.89 ± 0.03	0.998	1.89 ± 0.30	0.930	0.8	1.2	1.71 ± 0.05	0.955	0.8	1.2	0.30 ± 0.03	0.493	0.24	0.36

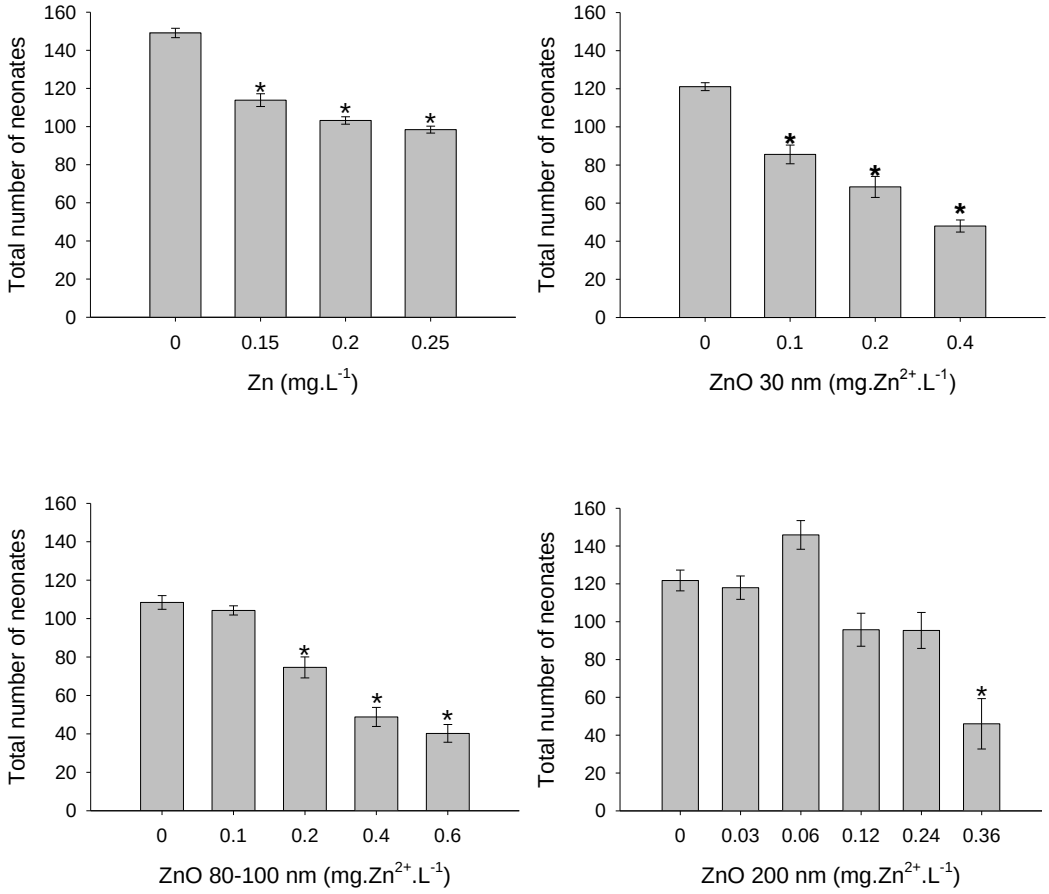

Fig. 2.3. Effects of Zn, ZnO-NPs (30 and 80-100 nm) and ZnO micro-sized in the number of neonates produced by *D. magna*. Data is expressed as mean values ± standard error. (*) Statistical differences at $p<0.05$.

The length of adult females at the end of 21d of exposure showed statistical differences between control and the higher test concentration (0.25 mg.Zn^{2+}.L^{-1}) of $ZnCl_2$ (Kruskal-Wallis one way ANOVA, H=12.74, df=4, p=0.005, Dunn's method, p<0.05), between control and the highest concentration (0.6 mg.Zn2+.L^{-1}) of ZnO-NPs 80-100n (Kruskal-Wallis one way ANOVA, H=14.66, df=4, p=0.005, Dunn's method, p<0.05) and between control and the concentrations 0.12, 0.24 and 0.26 mg.Zn^{2+}.L^{-1} for ZnO micro-sized (Kruskal-Wallis one way ANOVA, H=36.57, df=5, p≤0.001, Dunn's method, p<0.05) (Fig. 2.4). No statistical differences on the length of adult daphnids in ZnO-NPs of 30 nm were observed among the treatments at the end of 21d of exposure (Fig. 2.4).

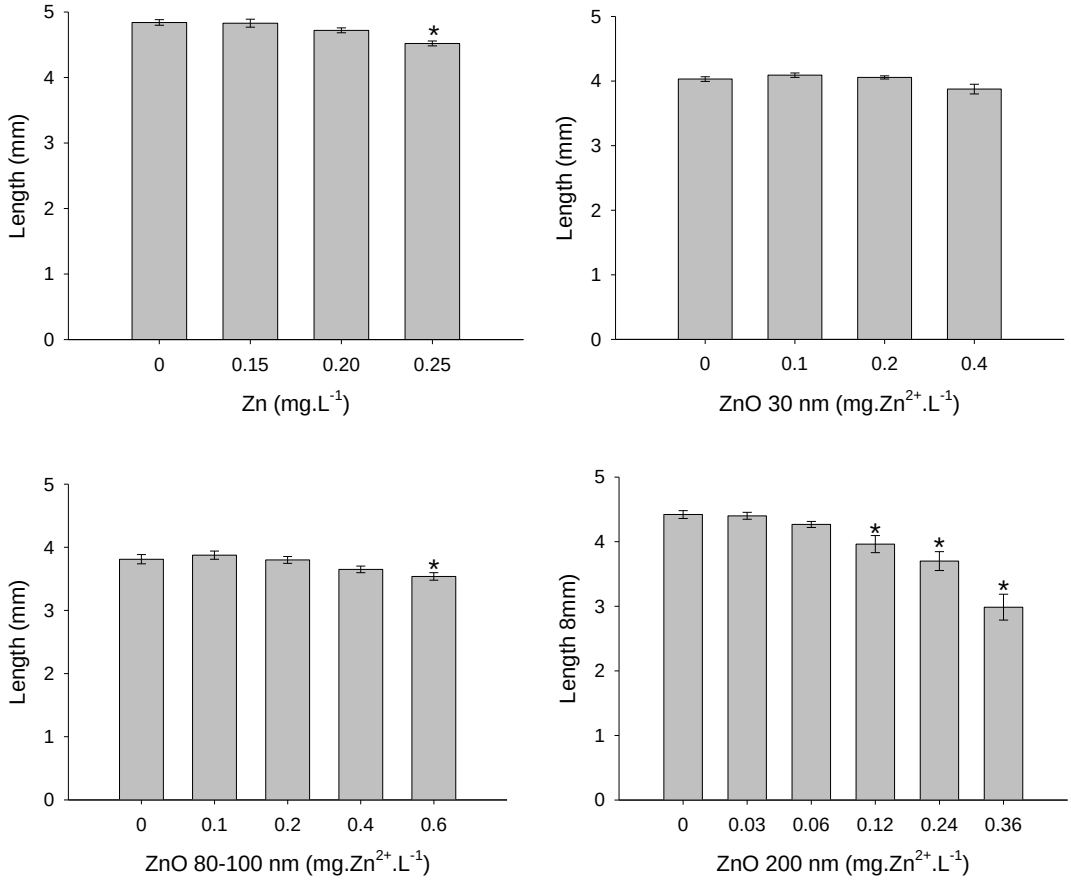

Fig. 2.4. Body length of 21d old *Daphnia magna* after exposure of Zn, ZnO-NPs (30 and 80-100 nm) and ZnO micro-sized. Data is expressed as mean values ± standard error. (*) Statistical differences at $p<0.05$.

Contrary to what happened on the immobilisation tests, the effect of $ZnCl_2$ on reproduction did not allow us to observe a complete dose-response relationship (EC_{50}> 0.25 mg.Zn^{2+}.L^{-1}). Concentrations inducing effects on reproduction or mortality along 21 days showed to have a small interval between them (> 0.25 mg.Zn^{2+}.L^{-1}; < 0.4 mg.Zn^{2+}.L^{-1}) (Table 2.1).

3.5. Discussion

The acute toxicities of ZnO-NPs 30 nm, 80-100 nm and the corresponding bulk material showed to be similar (1.02, 1.10 and 0.89 mg.Zn^{2+}.L^{-1}, respectively).

Previous studies have already reported similar toxicity between nanoscale ZnO and the respective bulk counterpart (Zhu et al., 2008; Zhu et al., 2009b; Yu et al., 2011). In addition Zn^{2+} (in the form of $ZnCl_2$) showed to be more toxic to *D. magna*, with an LC_{50}

value of 0.76 mg.Zn^{2+}.L^{-1}, when compared with ZnO nanoparticles. Acute toxicity of zinc (based on dissolved zinc concentrations) found in the literature indicates immobilisation 48h-EC_{50} values for cladocerans species, including *D. magna*, ranging between 0.38 – 4.31 mg.L^{-1} (Muyssen et al., 2005), going in accordance with our results.

The LC_{50} values for the immobilisation endpoint of the two different nanoparticles tested (30 nm and 80-100 nm) showed to be similar and not particle-size dependent (Table 2.1.). This is not in agreement with previous studies where smaller sized NPs were likely to cause higher toxicity because they may be taken up by organisms or accumulate within cells much easier due to its higher reactivity (Peng et al., 2011).

Particle size plays an important role in the toxicity of nanoparticles (Nowack and Bucheli, 2007; Bystrzejewska-Piotrowska et al., 2009). Therefore, the toxicity of ZnO-NPs should be more evident when compared with bulk form of the same compound (Yu et al., 2011). However our results showed that ZnO micro-sized was slightly more toxic when compared with ZnO-NPs of 30 and 80-100 nm (Table 2.1).

It is known that NPs are susceptible for aggregation in aqueous suspensions which may change their physicochemical properties making them less available to cause toxicity (Wong et al., 2010; Zhou and Keller, 2010). Therefore, the NPs tested (30 nm and 80-100 nm) likely could have suffered aggregation preventing their entrance across cell membranes limiting like this their toxic potential.

Reproduction tests with *D. magna* showed that the number of offspring produced was a more sensitive variable when compared with the endpoint immobilisation, showing a dose-response relationship with the concentrations tested. Like with the immobilisation endpoint, it was expected that smaller NPs would cause higher toxicity as theoretically smaller particles are more likely to be taken up by the filtering appendices of daphnids, interact with cell membranes and deposit in target organs (Nel et al., 2006). But this was not observed in our results as a key factor for the reproduction of *D. magna*.

No published data about chronic toxicity of ZnO-NPs to the cladoceran *D. magna* was found in the literature for comparison. However, in a similar study, Wiench et al. (2009), tested the toxic effect of coated TiO_2 NPs on the reproduction of *D. magna*. For instance, the authors showed that the reproduction endpoint was far more sensitive when compared with the acute tests, showing effects at 10-fold lower concentrations than effects on mortality. In the present study, differences on endpoints were not so high, varying between 4-fold (30 nm) and around 3-fold (80-100 nm and 200 nm). Giving the scarce studies concerning long-term effects of NPs, it becomes difficult to assess,

compare and understand the chronic effects of different nanoscale materials to aquatic organisms.

In addition, the LC_{50} values for $ZnCl_2$ for the 48h (without food) and the 21days (within the chronic assay) were 0.76 mg.Zn^{2+}.L^{-1} and > 0.25 < 0.40 mg.Zn^{2+}.L^{-1}, respectively (Table 2.1).

Reproduction upon $ZnCl_2$ exposure was adversely affected by the range of concentrations used. Sánchez-Ortíz et al., (2010) also showed a decrease in population growth in two cladocerans species (*Ceriodaphnia dubia* and *Daphnia pulex)* with increasing concentrations of zinc in the medium. The range of concentrations used by the authors ranged between 0.125 – 1 mg.L^{-1}, which are similar to the concentrations for our study (0.15 – 0.40 mg.Zn^{2+}.L^{-1}). In the last concentration (1 mg.L^{-1}) the authors observed no reproduction and dead after a week and an adverse effect of $ZnCl_2$ at a concentration (or higher) than 0.25 mg.L^{-1}.

A similar pattern was observed in our results. Around the 2nd week of exposure to $ZnCl_2$, daphnids showed low reproduction rates and started to die in all the replicates of the last three concentrations used (0.30, 0.35 and 0.40 mg.Zn^{2+}.L^{-1}), thus not being included in the statistical approach. Also, in the same study performed by the same authors, in the lowest concentration (0.125 mg.L^{-1}) for *D. pulex,* the population growth was higher than in control. In the data for chronic exposure of $ZnCl_2$ our results didn't show hormesis (stimulatory effect of sub-lethal concentrations).

These results show that in spite the fact zinc is an essential metal for all living organisms, toxicity at concentrations above the limit of Zn necessities for the organisms occurs, as it was shown in the three last concentrations tested (Muyssen et al., 2006). For the ZnO-micro sized we observed a stimulation of the number of offspring (hormesis) produced in the concentration of 0.06 mg.Zn^{2+}.L^{-1}. However, we cannot affirm that this is due to a low and beneficial concentration of zinc since we did not know the amount of zinc dissolved in the solution in the concentrations tested. The result obtained could be explained by aggregation events that lead to a decrease in the toxicity.

For the feeding inhibition endpoint, as it was observed for the other endpoints assessed, the feeding rates of *D. magna* showed a dose dependency with ZnO-NPs, ZnO micro-sized and $ZnCl_2$ concentrations.

A recent research on *D. magna* suggested that exposures of zinc via waterborne resulted in whole-body zinc burden and consequently leads to a decrease of Ca^{2+} body contents due to the competition between these two ions for ionoregulatory surfaces

(Evens et al., 2011). The depletion of Ca^{2+} levels (hypocalcemia) inhibits the filtration rates of daphnids leading inevitably to a decrease of food uptake (Muyssen et al., 2006). As a consequence, less energy will be available to allow a normal growth rate and reproduction. (Muyssen et al., 2006; Evens et al., 2011). These findings could then explain the results obtained in our study for the low feeding rates and the reproduction output of daphnids at the highest concentrations tested since during the reproduction tests, we observed that after the 2^{nd} week of exposure daphnids visibly showed difficulty to change their moults (except on the control) and consequently died (data not shown). Therefore we can hypothesize that zinc in excess can indirectly affect the moulting process of crustaceans, including *D. magna*.

A gene expression study showed that zinc (in the form of $ZnCl_2$) was responsible for the down regulation of several genes involved in the secretion of digestive enzymes. If low enzyme activity in the gut epithelium of daphnids occurs, nutrients will be poorly absorbed, leading to repercussions at the reproduction level (Evens et al., 2011). Another study showed that zinc ions were involved in the downregulation of two genes involved in the chitinase activity (Poynton et al., 2006). Chitin has an important role in the maintenance of exoskeleton and moulting processes of crustaceans. Concentrations of 0.1 $mg.L^{-1}$ were sufficient to cause a decrease of 20% in the chitinase activity (Poynton et al., 2006). Since daphnids need to shed their moult in order to give another brood of neonates and zinc interferes with the moulting process, this will result in implications for the reproduction of *D.magna* due to the decrease of chitinase activity (Poynton et al., 2006).

Regarding the toxic effects of ZnO-NPs, several studies have suggested that the toxicity of these NPs is mainly due to the dissolution of Zn^{2+} ions from NPs suspensions (Franklin et al., 2007; Heinlaan et al., 2008). However, this assumption is not consensual since other studies believe that the dissolution of Zn ions does not account for the total observed toxicity of ZnO-NPs (Zhu et al., 2008).

For instance, Poyton et al. (2011) in a study with *D. magna* demonstrated that both ZnO-NPs and Zn ions (from $ZnSO_4$) at sublethal concentrations contribute for the toxic effects on daphnids. However, authors suggested that both compounds have distinct modes of action since in the exposure concentrations of both compounds to *D. magna* only four genes overlapped in the gene expression profiles of both compounds. Therefore,

based on these results, the toxic effects caused by Zn ions and ZnO-NPs may be through different mechanisms of action.

These findings may then explain the panoply of the results found in the literature regarding the toxicity of ZnO-NPs to aquatic organisms.

Furthermore, even if in our study the EC_{50} values of all zinc compounds on the endpoints assessed were calculated as Zn^{2+} concentrations, it is important when dealing with metal-based nanoparticles to take into consideration its dissolution kinetics in order to verify if the dissolution of Zn^{2+} from ZnO nanoparticles ($ZnO + H_2O = Zn^{2+} + 2OH^-$) is immediate or if it takes time to reach an Zn^{2+} free equilibrium in aqueous dispersions. According David et al., (2012) ZnO nanoparticles solubility in Mili-Q water of different particle sizes (20 nm and 71 nm) and the respective bulk counterpart presented similar solubility kinetics and reached an equilibrium within 1h.

3.6. Conclusion

From our results we conclude that $ZnCl_2$ was more toxic to *Daphnia magna* on the immobilisation endpoint. However, for the feeding inhibition, ZnO-NPs showed to induce higher toxicity. Regarding the effects on the offspring production, it was observed that ZnO-NPs induced higher toxicity and that $ZnCl_2$ shows a toxicity pattern ranging from sublethal to lethal effects within a small gap in concentration.

In this study differences in the initial size of nanoparticles did not play a role on toxicity. This may be explained by their possible similarity in sizes in ASTM media but that has still to be confirmed by further particles' characterization.

Nevertheless, greater efforts in (nano)toxicological researches are needed to help stakeholders to create regulations and a sustainable nanotechnology industry.

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Chapter 4

General discussion and conclusions

4. General discussion and conclusions

4.1. General discussion and conclusions

Since the 1990's the production of nanoproducts has increased exponentially leading to environmental and health exposures (Bystrzejewska-Piotrowska et al., 2009). According with many authors, aquatic systems are considered carriers for ENPs upon their release into the environment.

Metal oxide nanoparticles, such as ZnO, TiO_2 and CeO_2, are more and more incorporated in consumer products (Keller et al., 2010). Consequently, sooner or later they will reach the environment.

There is already many available data in the literature regarding the toxic effects of this class of NPs to aquatic environments as shown in the first chapter.

However, the understanding on how NPs behave in water is not yet fully understood since their behaviour once released to the aquatic environment can be unpredictable and complex depending on several parameters (pH, ionic strength, ionic composition, NOM), which ultimately will control their aggregation and/or stability, affecting in turn their bioavailability (Keller et al., 2010; Sanchez et al., 2011).

The present study aimed to assess mainly the effect of ZnO-NPs to *Daphnia magna*, but also to present a brief overview of the effects of ZnO-NPs to other aquatic organisms.

On the second chapter it was shown that ZnO-NPs have a wide range of effects on aquatic organisms. Toxic effects are highly dependent on the test organism. Therefore, it is important to perform toxicity tests in a wide range of aquatic organisms (Barrena et al., 2009) to allow a perspective of the present and future effects in the food chain.

The magnitude of the thread may also depend on whether NPs are dispersed in the environment or in aggregates. However, this issue still presents limited information (French et al., 2009).

The mechanisms of toxicity associated with ZnO-NPs to organisms are thought to be the influence of the Zn^{2+} ions released into the solution and ROS production mainly due to their small particle size (Miller et al., 2010).

The third chapter addressed the effects of ZnO-NPs with different particle size to *Daphnia magna*, which is one of the most used organisms in toxicity testing.

For the immobilisation endpoint, the results showed similar patterns between the effect-dose values of all the ZnO-NPs tested. At the nanosize range, nanoparticles presented different physical and chemical properties that may lead to an increase of toxicity when compared with the bulk counterpart (Heinlaan et al., 2008). However in our results the 48h-EC_{50} values for the immobilization endpoint for both ZnO-NPs and ZnO micro-sized were similar. These results go in accordance to what was described by some authors on the similarity of toxicities between ZnO-NPs and the respective bulk (Fairbairn et al., 2011).

Heinlaan et al., (2008) using a recombinant sensor bacteria reported that at concentrations lower than 1 $mg.L^{-1}$, in average, 83% of nano and bulk ZnO was dissolved into Zn^{2+}. Therefore, according to the EC_{50} values for ZnO-NPs and ZnO micro-sized and taken into account the similarity of the EC_{50} value of $ZnCl_2$ (0.76 $mg.L^{-1}$), the toxicity for these NPs may be due to dissolved Zn^{2+}. However no chemical analysis data to determine nanoparticles dissolution is yet available, and therefore this cannot be inferred.

For the results of *D. magna* feeding behaviour, the ingestion of ZnO-NPs contaminated media may have caused a state of hypocalcaemia. According to Evens et al., (2011) the disturbance in the Ca^{2+} balance due to the exposure to Zn^{2+} can present an explanation for the low feeding rates observed by daphnids, but also for the reduced growth and offspring production.

Regarding the reproduction tests, the results also showed a dose level dependency. Sub-lethal effects including reduction of offspring production and body shrinkage of adult daphnids may cause considerable population-level impacts as well for the surrounding community since planktonic invertebrates are the food source for higher organisms in the food chain, such as fishes.

The NOEC values of ZnO-NPs obtained in our results ranged between 0.1 – 0.24 $mg.Zn^{2+}.L^{-1}$. In a study, Wiench et al., (2009) reported that the NOEC for the reproduction of *D. magna* after 21d under exposure of TiO_2 was of 3 $mg.L^{-1}$. This comparison may suggest that ZnO-NPs cause higher toxicity to *D. magna*.

Nanoparticles pose many challenges for the scientific community. Therefore, urges the need to catch up the rapid development of nanotechnology by addressing reliable guidelines and environmental quality standards (Elsaesser and Howard, 2011).

Even if currently there is already a lot of attention on the effects and behaviour of NPs in the environment, the state of knowledge relative to the toxic effects of NPs to aquatic organisms still presents some gaps that need to be addressed in the near future.

Deeper understanding concerning the fate, uptake, behaviour (aggregation/solubility/stability/adsorption) and bioavailability of NPs in aquatic environments should be assessed before nanowaste reaches the environment.

Moreover, long-term exposures should be widely performed for NPs toxicity assessments and not only rely to short-term effects with the purpose to induce a biological response. For instance, chronic exposures at low concentrations would give more realistic results since nanoparticles are not subject to degradation as soon they reach the environment (Ma et al., 2013); nanoparticles are likely to persist in the environment for long periods of time with possible interactions with organisms.

This fact is shown in our study where the reproduction (long-term exposure) endpoint was more sensitive than the other endpoints assessed (short-term exposures).

4.2. References

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